Impact of CYP3A5 Genetic Polymorphism on the Biotransformation of Drugs and Environmental Toxins

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AFBO	Aflatoxin B1-8,9-epoxide
AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
AFQ1	Aflatoxin Q1
BE	Baculovirus-expressed
B5	Cytochrome b5
BSA	Bovine Serum Albumin
CLp	Predicted pharmacokinetic clearance obtained by use of the well-stirred (CLp_1) and the parallel tube (CLp_2) models
CYP	Cytochrome P450
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FMN	Flavin mononucleotide
FAD	Flavin adenine dinucleotide
GSH	Reduced L-Glutathione
HLM	Human liver microsomes
HPLC	High performance liquid chromatography
K_m	Michaelis-Menten constant
LC-MS/MS	Liquid chromatography tandem mass spectrometry
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaCl	Sodium chloride
NQO1	NADPH quinone oxidoreductase
OR	NADPH-cytochrome P450 reductase
OR/b5	Baculovirus-expressed oxidoreductase with cytochrome b5
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
Rpm	Rounds per minute
SDS	Sodium Dodecyl Sulfate
TEMED	N, N, N', N'- Tetramethylethylenediamine
Tris	Tris-hydroxymethyl-aminomethane
V_{max}	Maximum reaction velocity

1 INTRODUCTION

All organisms are constantly and unavoidably exposed to foreign chemicals, so called xenobiotics, which include both synthetic and natural chemicals such as drugs, industrial chemicals, pesticides, and pollutants, pyrolysis products in cooked food, alkaloids, secondary plant metabolites, and toxins produced by molds, plants and animals. The physical property that enables many xenobiotics to be absorbed through the skin, lungs, or gastrointestinal tract, namely their lipophilicity, is an obstacle to their elimination because lipophilic compounds can either not be excreted via the bile or the kidney at all, or would be readily reabsorbed from the gut or the renal tubuli. Consequently, the elimination of xenobiotics often depends on their conversion to more water-soluble compounds by a process known as biotransformation, which is catalyzed by drug metabolizing enzymes (DMEs) in the liver and other tissues.

1.1 Drug Metabolizing Enzymes (DME)

Reactions catalyzed by DMEs are often divided into “Phase I” and “Phase II” (Table 1). Phase I DMEs, many of which are cytochromes P450, sometimes participate in detoxification of reactive substrates. In addition, they are often involved in the activation of inert protoxicants, promutagens and procarcinogens to electrophilic intermediates that can bind as adducts to proteins or DNA and/or cause oxidative stress (Dalton et al., 1999; Kidd et al., 1999; Nebert, 2000). Phase II DMEs (e.g. methyltransferases, UDP glucoronosyltransferases, glutathione transferases, sulfotransferases) are sometimes involved in metabolic activation (Nebert et al., 1996), but they usually conjugate various Phase I products and other reactive intermediates to form water-soluble derivatives, completing the detoxification cycle. Therefore, it seems likely, that genetic differences in the regulated expression of activity level of Phase I and Phase II DME genes might be crucial factors in defining susceptibility to toxicity or cancer caused by drugs and other environmental pollutants. Hundreds of genes coding for drug metabolizing enzymes exist in the human genome. Polymorphism in several such genes causing high levels of one enzyme and low levels of another enzyme in a specific pathway involved in the

metabolism of a particular environmental pollutant could lead to 30- or more than 40-fold differences between two individuals in response to that foreign chemical (Nebert, 2000).

Table 1*. Phase I and phase II DMEs

Enzyme class	Reaction type	Enzymes
	Phase I DMEs	
Oxidation	Hydroxylation, N- and O-dealkylation, desamination, oxidative dehalogenation	Cytochrome P450-monooxygenase
	N- and S-Oxidation	Cytochrome P450-monooxigenases, flavinmonooxigenases
	Dehydration	Alcohol dehydrogenases
	Dehydration of amines	Monoaminoxidases
Reduction	Dehalogenation of nitro groups	Cytochrome P450-monooxigenases
	Carbonyl reduction	Carbonylreductases
Hydrolysis	Hydrolysis of epoxides	Epoxide Hydrolases
	Hydrolysis of esters	Carboxylesterases
	Hydrolysis of peptides	Peptidases
Others	Oxidation of superoxide anions	Superoxide dismutases
	Peroxidation	Glutathione peroxidases
	Phase II DMEs	
Conjugation	Glucuronosylation	UDP-glucuronosyltransferases
	Sulfation	Sulfotransferases
	Acetylation	O- and N-acetyltransferases
	Methylation	O-, N- and S-methyltransferases
	Glutathione S-conjugation	Glutathione S-transferases

*Adapted from Elke Störmer, Dissertation, Berlin, 2001.

1.2 Cytochrome P450 Enzymes

1.2.1 Discovery

The recognition of the existence of cytochrome P450 hemoproteins dates back to the late 1950s, when a carbon-monoxide-binding protein pigment was reported to be present in the endoplasmic reticulum of the liver (Garfinkel, 1958; Klingenberg, 1958), and to the later identification of the pigment as b-type cytochrome (Omura and Sato, 1962; Omura and Sato, 1964a; Omura and Sato, 1964b).
Shortly thereafter, the ability of cytochrome P450 to serve as terminal oxidase in the metabolism of steroid hormones (Estabrook et al., 1963) and xenobiotics (Cooper et al., 1965) was demonstrated. These observations were quickly followed by the recognition that multiple forms of cytochrome P450 exit in the fragments of endoplasmic reticulum, the microsomes (Lu et al., 1971; Guengerich et al., 1982a; Guengerich et al., 1982b; Guengerich et al., 1982c).

1.2.2 Function

All cytochrome P450 enzymes are heme-containing proteins. The heme iron in cytochrome P450 is usually in the ferric (Fe^{3+}) state. When reduced to the ferrous (Fe^{2+}) state, cytochrome P450 can bind ligands such as O_2 and carbon monoxide (CO). The basis reaction catalyzed by cytochrome P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate (RH); the other one is reduced to water with reducing equivalents derived from NADPH:

$$O_2 + 2e^- + RH + 2H^+ \rightarrow H_2O + ROH$$

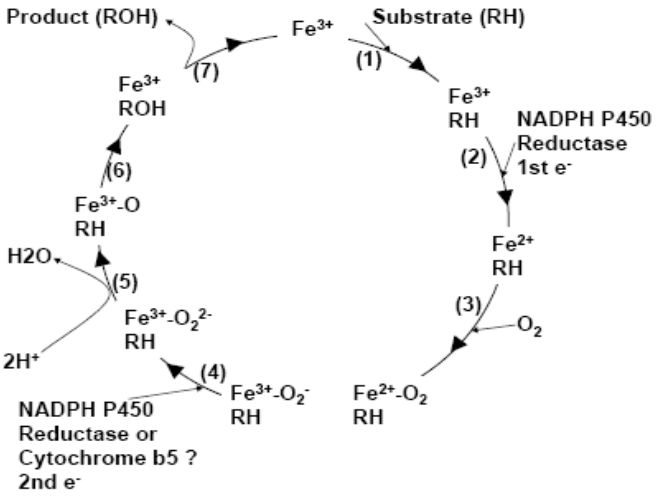


Fig. 1 Catalytic cycle of cytochrome P450 enzymes (adapted from Guengerich and Macdonald (Guengerich and MacDonald, 1990))

The catalytic cycle represented in the above picture may be summarized as follows: (1) The binding of a substrate to a P450 causes a lowering of the redox potential (Ruckpaul et al., 1985), which makes the transfer of an electron favorable from its redox partner, NADH or NADPH. This is accompanied by a change in the spin state of the hem iron at the active site. (2) The next stage in the cycle is the reduction of the Fe^{3+} ion by an electron transferred from NAD(P)H via an electron transfer chain that depends on a single specific NADPH P450 reductase that contains both FAD and FMN as cofactors (Coon et al., 1975). FAD can accept electrons from NADPH and FMN functions as the single electron carrier. (3) An O_2 molecule binds rapidly to the Fe^{2+} ion forming $Fe^{2+} + O_2$. There is evidence to suggest that this complex then undergoes a slow conversion to a more stable complex $Fe^{3+} - O_2^-$ (Archakov et al., 1990). (4) A second reduction is required by the stoichiometry of the reaction. This

has been determined to be the rate-determining step of the reaction (Imai et al., 1977). A comparison between the bond energies of O_2, O_2^-, and O_2^{2-} suggest that the Fe^{3+} - O_2^{2-} complex is the most favorable starting point for the next stage of the reaction to occur (Levis, 1996). Some microsomal P450s systems may receive the second electron from NADPH through cytochrome b5; however the mechanism of this interaction still remains speculative (Schenkman and Jansson, 2003).(5) The O_2^{2-} reacts with two protons from the surrounding solvent, breaking the O-O bound, forming water and leaving an (Fe – O)3+ complex. (6) The Fe-ligated oxygen atom (O) is transferred to the substrate forming an hydroxylated form of the substrate. (7) The product is released from the active site of the enzyme which returns to its initial state.

1.2.3 Evolution

Several suggestions concerning P450 evolution can be made based on the examination of the phylogenetic tree and its correlation with catalytic activities of cytochrome P450s. The earliest P450s are those that now metabolize steroids and fatty acids. The fatty acid-metabolizing P450 IV family and the steroid-inducible P450 III genes diverged more than 1 billion years ago. The P450 I and P450 II gene families formed about 800 million years ago and these genes are now responsible for the metabolism of drugs and carcinogens (Nelson and Strobel, 1987).

1.2.4 Classification

The highest concentration of P450 enzymes involved in xenobiotic biotransformation is found in the endoplasmatic reticulum (microsomes) of the liver, but P450 enzymes are expressed in almost all tissues. The human microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 gene families, namely CYP1, CYP2 and CYP3. Liver microsomes also contain P450 enzymes encoded by the CYP4 gene family, the substrates of which include several fatty acids and eicosanoids but relatively few xenobiotics. A classification of all existing 57 human P450s based on substrate class is given in Table 2.

Table 2*. Classification of human P450s based on major substrate class

CYP Family	Steroids	Xenobiotics	Fatty acids	Eicosanoids	Vitamins	Unknown
1	1A1	1A1	1A1	1A2	1A1	
	1A2	1A2	1B1		1A2	
	1B1	1B1			1B1	
2	2A6	2A6	2A6	2B6	2A6	2A7
	2B6	2A13	2B6	2C8	2B6	2R1
	2C18	2B6	2C19	2C9	2C19	2S1
	2C19	2C8	2D6	2E1	2D6	2U1
	2D6	2C9	2E1	2J2	2E1	2W1
	2E1	2C18	2J2			
	2J2	2C19				
		2D6				
		2E1				
		2F1				
		2J2				
3	3A4	3A4	3A4	3A4	3A4	3A43
	3A5	3A5	3A5		3A5	
	3A7	3A7			3A7	
	3A43					
4	4B1	4B1	4A11	4A11		4A22
		4F12	4B1	4F2		4F11
			4F2	4F3		4F22
			4F3	4F8		4V2
			4F8	4F12		4X1
			4F12			4Z1
5		5A1		5A1		
7	7A1					
	7B1					
8	8A1	8A1		8A1		
	8B1					
11	11A1	11A1			11B1	
	11B1	11B1				
	11B2	11B2				
Others	17	17			19	20
	19	19			24	26C1
	21A2	21A2			26A1	27C1
	27A1	26A1			26B1	
	39A1	51			27A1	
	46A1				27B1	
	51					

(*Adapted from Guengerich FP (Guengerich, 2004))

1.2.5 Interindividual variability

The expression and activity of each P450 enzyme have been shown to vary from one individual to the other, due to environmental and genetic factors (Meyer, 1994; Shimada et al., 1994). Decreased P450 activity can result from (1) a genetic mutation that either blocks the synthesis of a P450 enzyme or leads to the synthesis of a catalytically compromised or inactive enzyme, (2) exposure to an environmental factor (such as an infectious disease or a xenobiotic) that down-regulates P450 enzyme

expression, or (3) exposure to a xenobiotic that inhibits or inactivates a pre-existing P450 enzyme. By inhibiting cytochrome P450, one drug can impair the biotransformation of another drug. Such drug-drug interactions can lead to an excessive pharmacological or toxicological response to the second drug. In this regard, inhibition of cytochrome P450 mimics the effects of a genetic deficiency in P450 enzyme expression. Increased P450 enzyme activity can result from (1) gene duplication leading to overexpression of a P450 enzyme, (2) exposure to environmental factors, such as xenobiotics, that induce the synthesis of cytochrome P450, or (3) stimulation of a pre-existing enzyme by a xenobiotic. By inducing cytochrome P450 one drug can stimulate the metabolism of a second drug and thereby decrease or increase its therapeutic effect. A dramatic effect of this type of drug interaction is the induction of ethinylestradiol metabolism by phenobarbital and rifampin, which can decrease the contraceptive effect of the former drug and lead to pregnancy (Breckenridge et al., 1980). Similarly, a significant decrease (67%) in oral tacrolimus area under the concentration-time curve has been observed in healthy volunteers when co-administered with rifampin (CYP3A inducer) (Christians et al., 2002). Allelic variants, which arise by point mutations in the wild-type gene, are another source of interindividual variation in P450 activity. Amino acid substitution can increase or, more commonly, decrease P450 enzyme activity, although the effect may be substrate-dependent. Some of the genetic factors that influence P450 activity identified thus far are summarized ((Nagata and Yamazoe, 2002), http://www.imm.ki.se/CYPalleles). The environmental factors which are known to affect P450 expression include medications (e.g. barbiturates, rifampin, isoniazid), food (e.g. cruciferous vegetables, charcoal-broiled beef), social habits (e.g. alcohol consumption, cigarette smoking), and disease status (diabetes, inflammation, hyperthyroidism and hypothyroidism). When environmental factors influence P450 enzyme levels, a considerable variation may be observed when xenobiotic biotransformation (e.g. drug metabolism) is measured repeatedly in the same individual.

1.2.6 Clinical relevance of genetic polymorphisms on drug metabolism and disposition

Most DMEs exhibit clinically relevant genetic polymorphisms. Adverse drug reactions are common; they are responsible for a number of debilitating side effects and are a significant cause of death following drug therapy (Lazarou et al., 1998). It is now clear that a significant proportion of these

adverse drug reactions, as well as therapeutic failures, are caused by genetic polymorphisms, genetically based interindividual differences in drug absorption, disposition, metabolism, or excretion. Most of the commercially available drugs are metabolized by the phase I cytochrome P450 superfamily of DMEs. The clinical relevance is best-characterized for the genetic polymorphisms in CYP2D6, CYP2C19 and CYP2C9 (Stormer et al., 2000; Xie et al., 2004). CYP2D6 play important roles in the metabolism of beta-blockers, tricyclic antidepressants, antiarrythmic agents, antipsychotic agents and opioids. CYP2C19 is involved in the metabolism of proton-pump inhibitors whereas CYP2C9 metabolizes antidiabetics and anticoagulants.

1.3 CYP3A in drug metabolism

1.3.1 Expression and Variability

CYP3A is considered to be the most important drug-metabolizing enzyme subfamily in the human body as it is responsible for the metabolism of 45-60% of currently used drugs (Fig. 2), as well as many steroids, environmental chemicals, and carcinogens (Shimada et al., 1994; Li et al., 1995; Thummel et al., 1996; Rebbeck et al., 1998; Evans and Relling, 1999; Guengerich, 1999).

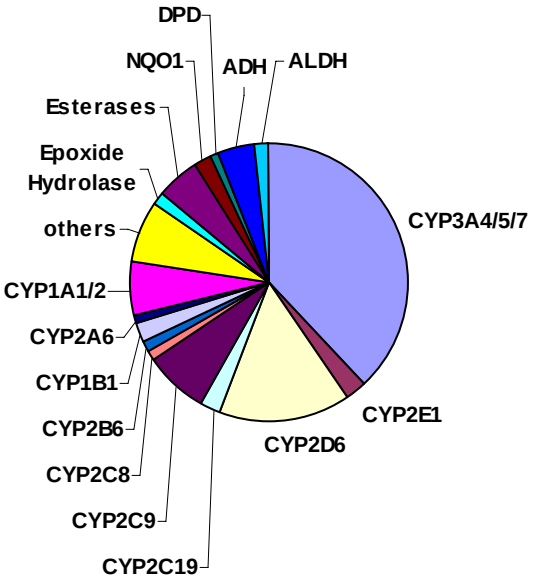


Fig.2 Relative contribution of specific enzymes to Phase I drug metabolism.

The percentage of Phase I metabolism of drugs contributed by each enzyme is estimated by the relative size of each section of the chart. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH: quinone oxidoreductase (adapted from (Evans and Relling, 1999)).

The members of this enzyme subfamily are the most abundantly expressed CYP enzymes in the liver (30% or more of total CYP content) (Shimada et al., 1994), small intestinal tissue (Kolars et al., 1992; Kolars et al., 1994; Lown et al., 1994; Paine et al., 1997; Koch et al., 2002; Lin et al., 2002), and kidney (Schuetz et al., 1992; Haehner et al., 1996; Koch et al., 2002; Givens et al., 2003). The wide substrate spectrum of CYP3A is the reason behind their frequent involvement in drug-drug interactions. Drug interactions may reduce CYP3A metabolic activity through inhibition or may increase it through induction. Such interactions can expand the range of variability of the area under curve (AUC) for CYP3A substrates to about 400-fold (Thummel and Wilkinson, 1998; Levy et al., 2001). However, the marked interindividual differences in CYP3A activity have also been reported to reflect genetic components (Ozdemir et al., 2000). Therefore, there has been a considerable effort to identify CYP3A gene mutations which might affect the expression and function of the CYP3A enzymes.

The human CYP3A locus is comprised of four functional genes (CYP3A4, CYP3A5, CYP3A7 and CYP3A43), but the differentiation between their products has proven difficult, due to the similarities in their protein sequence, in antigenic properties and due to overlapping substrate specificities (Gellner et al., 2001). In consequence, even though the variability in the expression is established for the three most important CYP3A genes (CYP3A4, CYP3A5 and CYP3A7), their respective contributions to the hepatic CYP3A pool and their effects on drug metabolism are still a matter of debate.

Table 3. Contribution of each CYP3A enzymes to the total hepatic CYP3A protein pool

CYP3A enzymes	Contribution to the total hepatic CYP3A protein pool (%)	References
CYP3A4	40-98%	(Wrighton et al., 1990; Tateishi et al., 1999; Kuehl et al., 2001; Koch et al., 2002; Lin et al., 2002; Westlind-Johnsson et al., 2003)
CYP3A5	2-60%	
CYP3A7	13%, 24%	(Stevens et al., 2003; Sim et al., 2005)
CYP3A43	Not detected	(Domanski et al., 2001; Gellner et al., 2001; Westlind et al., 2001)

1.3.2 CYP3A polymorphisms

The four CYP3A genes encoding their respective enzymes are localized in a 231-kb cluster on chromosome band 7q21-q22.1 (Brooks et al., 1988; Spurr et al., 1989; Inoue et al., 1992), and reside in tandem, adjacent to each other in the order: CYP3A43-CYP3A4-CYP3A7-CYP3A5 (Nelson et al., 1996; Domanski et al., 2001; Gellner et al., 2001; Finta and Zaphiropoulos, 2002); in which the CYP3A43 gene is in a head-to-head orientation with its neighboring gene CYP3A4, and the other three genes lie in head-to-tail orientation. Two pseudogenes (Nelson et al., 2004), CYP3AP1 and CYP3AP2 are present between the intergenic regions of CYP3A7-CYP3A5 and CYP3A4-CYP3A7, respectively (Finta and Zaphiropoulos, 2000).

Table 4[*]. Ethnic distribution of variant alleles of CYP3A

CYP3A genetic variants	References	Allele frequencies (%) in different ethnic groups				
		African	Hispanic	Caucasian	Chinese	Japanese
	CYP3A4					
CYP3A4*1B	(Rebbeck et al., 1998; Walker et al., 1998; Paris et al., 1999; Sata et al., 2000; Kuehl et al., 2001)	35-67%	9.3-11%	2-9.6%	0%	0%
CYP3A4*2	(Sata et al., 2000)	0%	N.A.	2.7%	0%	N.A.
CYP3A4*3	(Sata et al., 2000)	N.A.	N.A.	0.47-4%	1.5%	N.A.
CYP3A4*4	(Hsieh et al., 2001)	N.A.	N.A.	N.A.	1.5%	N.A.
CYP3A4*5	(Hsieh et al., 2001)	N.A.	N.A.	N.A.	0.98%	N.A.
CYP3A4*6	(Hsieh et al., 2001)	N.A.	N.A.	N.A.	0.5%	N.A.
CYP3A4*7	(Eiselt et al., 2001)	N.A.	N.A.	1.41%	N.A.	N.A.
CYP3A4*8	(Eiselt et al., 2001)	N.A.	N.A.	0.33%	N.A.	N.A.
CYP3A4*9	(Eiselt et al., 2001)	N.A.	N.A.	0.24%	N.A.	N.A.
CYP3A4*10	(Lamba et al., 2002)	2%	5%	0.24-2%	N.A.	N.A.
CYP3A4*11	(Eiselt et al., 2001)	N.A.	N.A.	0.34%	N.A.	N.A.
CYP3A4*12	(Eiselt et al., 2001)	N.A.	N.A.	0.34%	N.A.	N.A.
CYP3A4*13	(Eiselt et al., 2001)	N.A.	N.A.	0.34%	N.A.	N.A.
CYP3A4*14	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
CYP3A4*15A	(Dai et al., 2001; Lamba et al., 2002)	2-4.2%	-	0%	0%	0%
CYP3A4*16	(Lamba et al., 2002)	N.A.	5%	N.A.	N.A.	5%
CYP3A4*17	(Dai et al., 2001)	0%	0%	2.1%	0%	0%
CYP3A4*18	(Dai et al., 2001)	N.A.	N.A.	N.A.	10%	N.A.
	CYP3A5					
CYP3A5*2	(Jounaidi et al., 1996; Hustert et al., 2001)	0%	N.A.	1.9-5%	N.A.	N.A.
CYP3A5*3	(Hustert et al., 2001; Kuehl et al., 2001)	27-50%	75%	70%	65-73%	71-85%
CYP3A5*5	(Chou et al., 2001)				0.9%	
CYP3A5*6	(Hustert et al., 2001; Kuehl et al., 2001)	13%	0%	0%	0%	0%
CYP3A5*7	(Hustert et al., 2001)	10%	0%	0%	0%	0%
CYP3A5*8	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

CYP3A5*9	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
CYP3A5*10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
	CYP3A7					
CYP3A7*1B	(Kuehl et al., 2001)	0%	N.A.	1%	N.A.	N.A.
CYP3A7*1C	(Kuehl et al., 2001; Burk et al., 2002)	6%		3%		
CYP3A7*1D	(Kuehl et al., 2001)	0%	N.A.	1%	N.A.	N.A.
CYP3A7*1E	(Kuehl et al., 2001)	8%	N.A.	0%	N.A.	N.A.
	CYP3A43					
CYP3A43*3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

* N.A., not available

1.3.2.1 CYP3A4 genetic variability

Identification of single nucleotide polymorphisms (SNPs) in the CYP3A genes has been an active area of research. Currently, 39 CYP3A4 alleles, comprising 65 SNPs have been reported (Human Cytochrome P450 (CYP) Allele Nomenclature Committee. CYP3A4 allele nomenclature. Available from URL: http://www.imm.ki.se/CYPalleles/cyp3a4.htm.). By far the most common CYP3A4 genetic variant is the A-392G transition (CYP3A4*1B) located in the 5'-regulatory region (Rebbeck et al., 1998; Westlind et al., 1999). CYP3A4*1B allelic frequency varies among different ethnic groups: 0% in Asian, 5% in Caucasians and 54% in Africans (Chowbay et al., 2005). The other allelic variants occur at much lower frequencies (<1%-2%) or they are selectively prevalent in specific populations (Hamzeiy et al., 2002; Lamba et al., 2002; Floyd et al., 2003).

Many studies have been carried out on functions of CYP3A4 variants. CYP3A4*1B has been studied to ascertain the effect of the mutation on transcriptional activity and *in vivo* catalytic activity (Amirimani et al., 1999; Ando et al., 1999). The results of studies with larger numbers of predominantly Caucasian liver samples demonstrate no clear association between CYP3A4*1B variant and CYP3A4 specific content or catalytic activity (Westlind et al., 1999; Lamba et al., 2002). Compared with wild-type enzyme, there was no significant difference in the rates of CYP3A4*3, CYP3A4*7, CYP3A4*9, CYP3A4*11 and CYP3A4*19 metabolizing the probe substrates testosterone, progesterone, or 7-benzyloxy-4-(trifluoromethyl) coumarin (Sata et al., 2000; Dai et al., 2001; Eiselt et al., 2001). It means that these variants have no pronounced effect on drug metabolism kinetics. The individuals with CYP3A4*8 and CYP3A4*13 genotypes may have lower CYP3A4 protein content, since these variants appear to affect steady-state enzyme levels by altering heme binding and/or protein stability (Eiselt et al., 2001). For CYP3A4*2, CYP3A4*10, CYP3A4*14, CYP3A4*15 and CYP3A4*16, there was no significant association with midazolam hydroxylation activity (Lamba et al., 2002). Those with CYP3A4*17 genotype exhibited *in vitro* a significantly lower turnover of testosterone and of the insecticide chlorpyrifos than those with CYP3A4*1, while those with CYP3A4*18 metabolized both substrates with a higher turnover (Dai et al., 2001). Subjects carrying CYP3A4*4, CYP3A4*5 or CYP3A4*6 exhibited below average 6 beta-hydroxycortisol to cortisol ratio, implying reduced catalytic activity for the corresponding protein variants (Hsieh et al.,

2001). Indeed, most of the changes in catalytic activity observed for CYP3A4 gene variants are relatively modest. These catalytic findings and consideration of the low allele frequencies for the known structural CYP3A4 variants, implies that they are not the major cause of interindividual differences in CYP3A-mediated drug clearance in the general population.

1.3.2.2 CYP3A5 genetic variability

The genetic basis of the CYP3A5 polymorphism has gradually been elucidated following the publication in 2000 by Paulussen and colleagues that demonstrated the existence of genetic variants in complete concordance with the polymorphic CYP3A5 expression in the liver (Paulussen et al., 2000). To date 10 CYP3A5 alleles, consisting of 22 SNPs, have been identified (Human Cytochrome P450 (CYP) Allele Nomenclature Committee. CYP3A5 allele nomenclature. Available from URL: http://www.imm.ki.se/CYPalleles/cyp3a5.htm.). Of the variants found, CYP3A5*3 (g.6986G) is the only one found in all ethnic groups tested. The frequencies vary from 27% in African-Americans to 95% in Caucasians (Hustert et al., 2001; Kuehl et al., 2001; Fukuen et al., 2002; van Schaik et al., 2002; Hu et al., 2005; Roy et al., 2005). Other variants such as CYP3A5*6 and CYP3A5*7 affecting the CYP3A5 expression are relatively frequent in African subjects (10-22%) but absent in white subjects (Roy et al., 2005). The remaining CYP3A5 genetic variants are rare or occur at much lower allelic frequencies (Lamba et al., 2002; Roy et al., 2005).

In contrast to CY3A4, CYP3A5 expression in human exhibits a bimodal distribution, with the proportion of CYP3A5 “high expressers” and “low expressers” varying depending on the ethnic background. These interethnic differences in the prevalence of the CYP3A5 polymorphism are in part caused by differing allelic frequencies of the major genetic determinant of this trait, the g.6986A>G polymorphism (Hustert et al., 2001; Kuehl et al., 2001). This variant is located in intron 3 of the CYP3A5 gene. The inheritance of an adenine at this position (g.6986A, CYP3A5*1) allows for normal generation of CYP3A5 transcripts, whereas a guanine (g.6986G, CYP3A5*3) generates a cryptic splice with the resulting degradation of CYP3A5 transcripts (Kuehl et al., 2001). In Caucasians the concordance between the presence of g.6986A and increased CYP3A5 expression is high. Among 183 liver samples tested, all 18 livers with increased CYP3A5 protein levels had at least one allele

with adenine at g.6986 (Hustert et al., 2001). In samples of African origin the situation is more complex with the g.6986A>G polymorphism being less predictive of CYP3A5 expression (Kuehl et al., 2001). This is because of additional variants specific to Africans such as adenine at the polymorphic position g.14690G>A (also referred as CYP3A5*6 allele). This polymorphism is observed in 13% of CYP3A5 gene loci of African origin and results in the skipping of CYP3A5 exon 7 transcription (Kuehl et al., 2001). The g.27131-27132insT (also referred to as CYP3A5*7 allele), which terminates the open reading frame of CYP3A5 at position 348, is found in approximately 10% of CYP3A5 gene loci in this ethnic group. Both these variants have been found in samples homozygous for g.6986A (Hustert et al., 2001). The functional significance of several CYP3A5 genetic variants has been investigated. CYP3A5*2 has been reported to be present in two out of five liver tissues not expressing CYP3A5 protein, implying that this variant may confer protein instability (Jounaidi et al., 1996; Kuehl et al., 2001) or may be in strong linkage disequilibrium with other currently unknown mutations responsible for impaired protein expression and stability. The CYP3A5*5 variant produces multiple splicing products in Caco-2 cell lines (Chou et al., 2001). CYP3A5*6 carriers showed lower CYP3A5 protein expression and catalytic activity in three liver samples from African-Americans compared with the CYP3A5*1 carriers (Kuehl et al., 2001). CYP3A5*7 is an insertion variant resulting in a premature stop codon and thus protein truncation (Chou et al., 2001; Hustert et al., 2001; Lee et al., 2003). *In vitro* functional data using purified P450s (Lee et al., 2003) showed that for both the intrinsic clearance (V_{max}/K_m) of testosterone and the V_{max} of nifedipine oxidation, the rank order was: CYP3A5*1> CYP3A5*9> CYP3A5*8> CYP3A5*10. Low expression of CYP3A5 mRNA and protein was found to be associated with the most frequent and functionally important CYP3A5*3 genotype, regardless of racial ancestry (Hustert et al., 2001; Kuehl et al., 2001).

1.3.2.3 CYP3A7 genetic variability

To date, 6 CYP3A7 alleles were available on the CYP3A7 allele nomenclature webpage (Human Cytochrome P450 (CYP) Allele Nomenclature Committee. CYP3A7 allele nomenclature. Available from URL: http://www.imm.ki.se/CYPalleles/cyp3a7.htm.). The most common CYP3A7 variant

reported is CYP3A7*2 which carries a mutation in exon 11 and occurs at a frequency of 8%, 17%, 28%, and 62% in white, Saudi Arabian, Chinese, and African individuals, respectively (Rodriguez-Antona et al., 2005). CYP3A7*1C allele was reported at a frequency of 3% and 6% in Caucasians and African-American, respectively (Kuehl et al., 2001; Burk et al., 2002). CYP3A7*1B and CYP3A7*1D was found to be 1% for Caucasians and was not detected in African-Americans (Kuehl et al., 2001). In contrast, CYP3A7*1E was only detected in African-Americans at a frequency of 8% (Kuehl et al., 2001).

CYP3A7, the predominant CYP3A isoform in fetal liver (de Wildt et al., 1999; Ring et al., 1999), is expressed polymorphically in livers of white individuals, with approximately 11% of samples belonging to a high-expression subgroup (Burk et al., 2002). Two-thirds of the subjects in this group carry either the CYP3A7*1C allele, or less frequently, the CYP3A7*1B allele, which have been originally described by Kuehl and colleagues (Kuehl et al., 2001). Fetal livers homozygous for CYP3A7*2 had similar or higher CYP3A7 protein contents than CYP3A7*1 livers (Rodriguez-Antona et al., 2005). Kinetic studies showed that CYP3A7*2 has significantly higher catalytic constant (K_{cat}) compared with CYP3A7.1 toward dehydroepiandrosterone (DHEA). Consistently, fetal livers expressing CYP3A7*2 had an increased metabolism of the CYP3A7-specific substrate DHEA (Rodriguez-Antona et al., 2005). By analogy to CYP3A5, CYP3A7 increases the activity and versatility of biotransformation pathways for drugs and other xenobiotics, as well as endogenous compounds; as it is also concomitantly expressed in the liver, intestine, adrenal gland, prostate and thymus of adults.

1.3.2.4 CYP3A43 genetic variability

CYP3A43 was the last CYP3A to be discovered (Domanski et al., 2001; Gellner et al., 2001; Westlind et al., 2001). Thus far, CYP3A43 has not been demonstrated to produce a protein *in vivo* and may in fact be a pseudogene, although a low level activity has been described for CYP3A43 protein expressed in an *E. coli* (Domanski et al., 2001). It was therefore surprising, when Zeigler-Johnson and colleagues (Loukola et al., 2004; Zeigler-Johnson et al., 2004) reported an association between prostate cancer risk in men with a family history of this disease and the missense variant *CYP3A43*3*. Even more

recently, homozygocity for the same variant has been associated with a 2.6-fold increase in prostate cancer risk in African-Americans (Stone et al., 2005). Interestingly, prostate expresses more CYP3A43 transcripts than most other human tissues (Gellner et al., 2001). It remains unclear if these associations reflect a genuine CYP3A43 effect and would be its mechanism. CYP3A43 has been reported to contribute exons to CYP3A4 and CYP3A5 transcripts via trans-splicing, although levels of the resulting chimeric molecules are probably very low (Finta and Zaphiropoulos, 2000). Since the CYP3A locus exhibits high linkage disequilibrium (Thompson et al., 2004; Zeigler-Johnson et al., 2004; Schirmer et al., 2006) the associations between *CYP3A43*3* and prostate cancer could alternatively reflect the effect of a variant in another CYP3A gene. Several other CYP3A43 gene variants have recently been reported in a French cohort, including two nucleotide substitutions, one silent mutation, one missense mutation, and a frame shift mutation (Cauffiez et al., 2004), but their functional or clinical effects at present are unknown.

1.4 Investigated known and presumed substrates of CYP3A enzymes

1.4.1 Testosterone

Testosterone, the principal androgen or male sex hormone is one of the groups of compounds known as anabolic steroids. Testosterone is secreted mainly by the testes but it is also synthesized in small quantities in the ovaries, cortices of the adrenal glands, and placenta, usually from cholesterol. Pharmacologically, testosterone is used in the treatment of hypogonadism, cryptorchism, carcinomas, and menorrhagia (Darby and Anawalt, 2005). In recent years, testosterone has been increasingly used as an anti-aging medication (Shomali, 1997).

The effects of testosterone (Fig. 3) in humans occur by way of two main mechanisms: by activation of the androgen receptor (directly or as 5α-dihydrotestosterone), and by conversion to estradiol and activation of certain estrogen receptors.

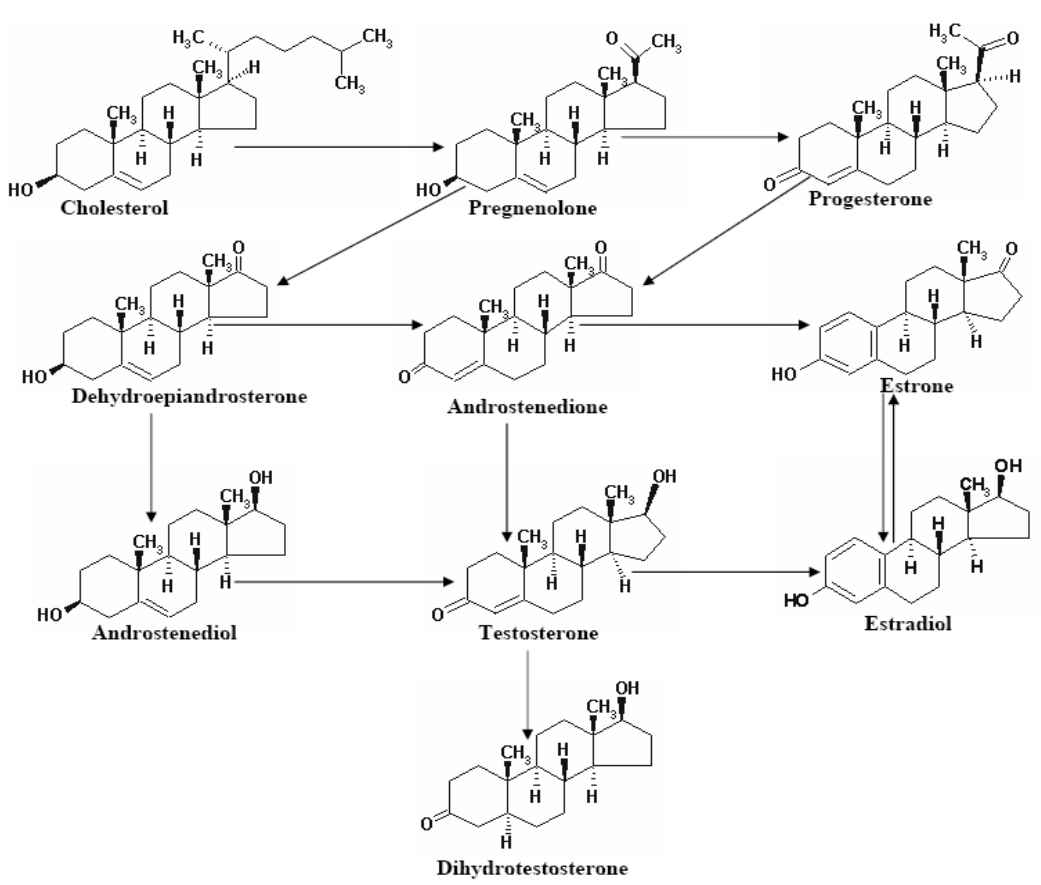


Fig. 3 Pathways for testicular androgen and estrogen biosynthesis

Free testosterone is transported into the cytoplasm of target tissue cells, where it can bind to the androgen receptor, or it can be reduced to DHT by the cytoplasmic enzyme 5α-reductase. DHT binds to the same androgen receptor even more strongly than testosterone, so that its androgenic potency is about 2.5 times that of testosterone (Grino et al., 1990).

Testosterone effects can be classified as virilizing and anabolic effects. Virilizing effects include growth of the penis, formation of the scrotum, and deepening of the voice, as well as beard and torso hair. Many of these effects fall into the category of secondary sex characteristics. When they occur to an unwanted degree in women they are termed "virilization" or "masculinization". Anabolic effects of testosterone include growth of muscle mass and strength, increased bone density and strength, and stimulation of height growth and bone maturation.

Testosterone 6ß-hydroxylation has long been recognized as a CYP3A-mediated reaction (Fig. 4). In HLMs numerous studies demonstrate that selective CYP3A inhibitors diminish the 6ß-hydroxylation of testosterone (Wrighton et al., 1990; Newton et al., 1995; Bourrie et al., 1996).

Specific antibodies against CYP3A inhibit the formation of 6ß-hydroxytestosterone by more than 90% (Gelboin et al., 1995; Mei et al., 1999; Shou et al., 2000). Studies with purified human proteins (Yamazaki and Shimada, 1997) and with cDNA-expressed enzymes (Waxman et al., 1991; Ono et al., 1996) provide additional evidence that all CYP3A members catalyze testosterone 6ß-hydroxylation. CYP2C9 and CYP2C19 also catalyze the reaction, but at 1/10 the rate of CYP3A (Yamazaki and Shimada, 1997). It was concluded that testosterone 6ß-hydroxylation rate primarily reflects the CYP3A activity, and thus can be used as a CYP3A *in vitro* phenotyping probe.

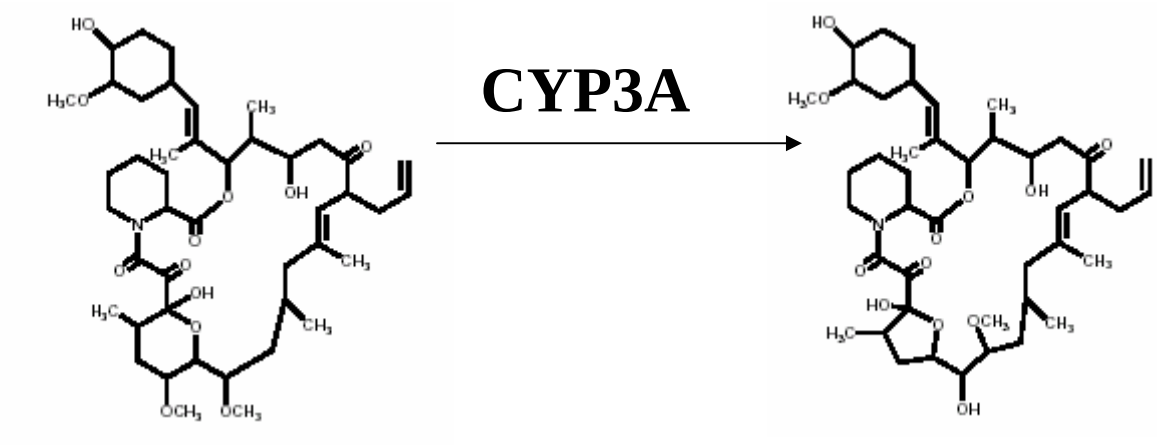
Fig. 4 CYP3A mediated testosterone 6ß-hydroxylation

1.4.2 Tacrolimus

Tacrolimus, a potent immunosuppressive macrolide lactone isolated from the fermentation broth of Streptomyces tsukabaensis (Goto et al., 1987), is a relatively specific inhibitor of T lymphocyte proliferation, which exerts its immunosuppressive activity mainly through binding to immunophilins (FK-binding proteins, FKBP). On account of these immunosuppressive properties, it is widely used in the prophylaxis of organ rejection after allogenic solid organ transplantation. Tacrolimus is subject to substantial intestinal and hepatic first-pass metabolism and its bioavailability is individually variable. Thus, tacrolimus elimination expressed as total body clearance varies inter-individually from 0.041 to 0.36 L/hr/kg of body weight (Christians et al., 2002). Because of this variability in conjunction with its narrow therapeutic index, monitoring of whole blood concentrations of tacrolimus is frequently used to achieve optimal efficacy while minimizing the risk of toxicity (Oellerich et al., 1998). Tacrolimus undergoes O-demethylation, hydroxylation and/or oxidative metabolic reactions in the liver and in the intestine, and it is eliminated mostly with the bile (Christians et al., 1991; Lhoest et al., 1991; Sattler et al., 1992; Vincent et al., 1992; Lhoest et al., 1994; Shiraga et al., 1994). Demethylation to 13-O-demethyltacrolimus by CYP3A is quantitatively the most important metabolic route (Fig. 5) (Sattler et al., 1992; Vincent et al., 1992; Karanam et al., 1994; Lampen et al., 1995).

Several clinical studies have demonstrated that carriers of CYP3A5*1 alleles (CYP3A5 “high expressers”) require higher doses of tacrolimus to achieve target blood concentrations than homozygous carriers of the CYP3A5*3 allele (CYP3A5 “low expressers”) (Macphee et al., 2002;

Hesselink et al., 2003; Thervet et al., 2003; Zheng et al., 2003; Haufroid et al., 2004; Macphee et al., 2005; Thervet et al., 2005), but it is not yet clearly understood, to what extend CYP3A5 contributes to tacrolimus biotransformation and why there is a clinically detectable difference although the CYP3A5 content relative to that of CYP3A4 is even in the high expressers of CYP3A5 only about ¼.



Tacrolimus **13-O-demethyltacrolimus**

Fig. 5 13-O demethylation of Tacrolimus by CYP3A

1.4.3 Aflatoxin B1 (AFB1)

AFB1 is produced by certain *Aspergillus* species, which contaminate human and animal food during growth and after harvest. The highest exposure to AFB1 has been observed in parts of Africa, China and Southeast Asia, which are also characterized by a high incidence of Hepatocellular carcinoma (HCC) (Wild and Hall, 2000). HCC is the most common type of liver cancer and the fifth most common cancer in the world (Wild and Hall, 2000). Main risk factors for HCC are hepatitis B and C viruses, exposure to aflatoxin B1 (AFB1). Positive association has been found between individuals with HCC and the presence of 249^{ser} mutation (AGG⇨AGT transversion at codon 249) in the tumor suppressor gene TP53 (Bressac et al., 1991; Hsu et al., 1991; Lasky and Magder, 1997; Montesano et al., 1997; Stern et al., 2001). Biotransformation is critical for the carcinogenic effect of AFB1. The four main products of AFB1 bioactivation are AFM1, AFQ1, AFB1-endo-8,9-epoxide and AFB1-8,9-exo-epoxide (Fig. 6). The last compound is the only known genotoxic derivative, which binds to DNA

to form the predominating 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1-N7-Gua) adduct (Wild and Turner, 2002). AFB1-N7-Gua confers the mutagenic properties of the compound. The other metabolites are poorer substrates for epoxidation and, consequently, are less mutagenic, carcinogenic and toxic than AFB1.

CYP3A and CYP1A2 are the major enzymes involved in the metabolism of AFB1 in adult human liver microsomes (Forrester et al., 1990; Ramsdell et al., 1991; Ueng et al., 1995; Patterson et al., 1999). CYP3A4 produces predominantly AFB1-8,9-exo-epoxide and AFQ1, whilst CYP1A2 leads to the formation of AFB1-8,9-exo and endo-epoxide, and of AFM1. However, there is still some discrepancy regarding the relative importance of CYP1A2 and CYP3A4 in human AFB1-8,9-exo-epoxide formation. CYP3A7 plays a role in the bioactivation of AFB1 to 8,9-exo-epoxide (Kitada et al., 1989; Kamataki et al., 1995; Yamada et al., 1998). CYP3A5 also metabolizes AFB1, however mainly to the exo-epoxide, with much less efficient formation of the detoxification product, AFQ1 (Wang et al., 1998). An association between increased levels of aflatoxin-albumin adducts and CYP3A5 “high expresser” status, particularly in individuals with low concomitant CYP3A4 expression, has been reported in AFB1-exposed Gambians (Wojnowski et al., 2004).

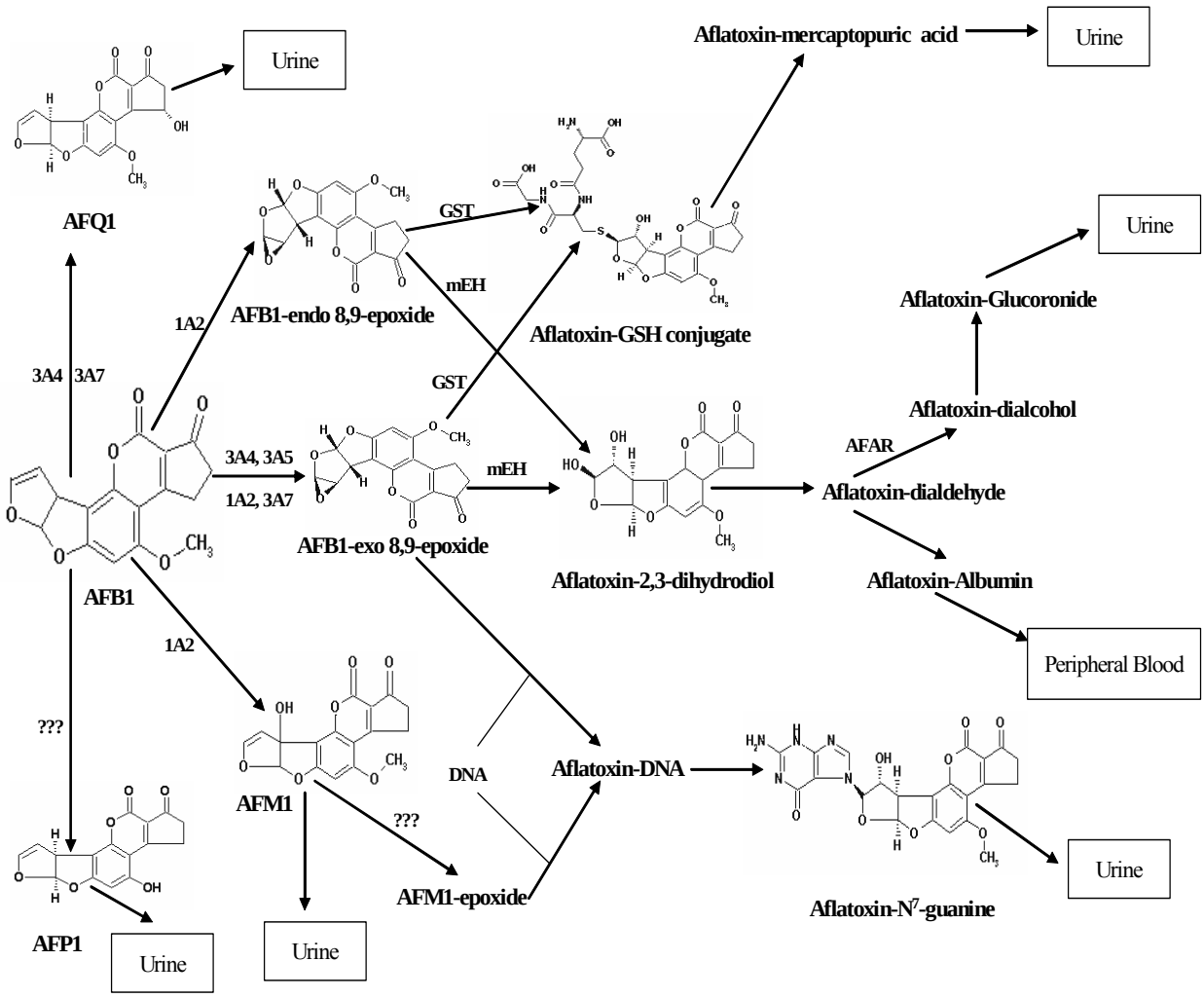


Fig. 6 Biochemical pathways of AFB1 metabolism in humans. 1A2, CYP1A2; 3A4, CYP3A4; 3A5, CYP3A5; 3A7, CYP3A7; GST, glutathione S-transferase; mEH, microsomal epoxide hydrolase; AFAR, aflatoxin aldehyde reductase (Adapted from Wild and Turner (Wild and Turner, 2002)).

2 AIMS OF THE STUDY

The aims of this thesis were to investigate the impact of CYP3A5 genetic polymorphisms for humans in health and disease. In this context, four specific questions should be answered:

1. To investigate the experimental determinants of CYP3A5 compared to CYP3A4 catalytic activity *in vitro* by optimizing the details of enzyme kinetic analysis using testosterone as an index substrate for CYP3A4 and CYP3A5 enzymes. In this context, different sources of artificially expressed CYP3A enzymes and cytochrome reductase and different ratios between CYP and reductase/cytochrome b5 should be compared.
2. To assess the relative contribution of CYP3A5 to the overall hepatic CYP3A activity among Caucasian. For this purpose, we used 6ß-hydroxylation of testosterone as a CYP3A specific probe, baculovirus-expressed CYP3A4 and CYP3A5 enzymes, microsomes isolated from 47 Caucasian livers with quantified amounts of CYP3A4 and CYP3A5 proteins, genotype-phenotype correlation analysis, as well as assessment of relative contributions based on the hepatic abundance approach.
3. To investigate the contribution of CYP3A5 to the *in vitro* hepatic metabolism of the macrolide immunosuppressive drug tacrolimus using baculovirus-expressed enzymes, microsomes derived from low and high CYP3A5 expressers, genotype-phenotype analysis, assessment of relative contribution based on the hepatic abundance approach and the prediction of tacrolimus pharmacokinetic clearance.
4. To evaluate the contribution of CYP3A5, as well as that of CYP3A4, CYP3A7 and CYP1A2 to the hepatic production of the reactive and carcinogenic aflatoxin B1 (AFB1) metabolite AFB1-8,9-epoxide using baculovirus-expressed enzymes, microsomes derived from low and high CYP3A5 expressers, genotype-phenotype analysis and assessment of relative contribution based on the hepatic abundance approach.

3 MATERIALS AND METHODS

To assess the impact of CYP3A5 genetic polymorphism on the biotransformation of drugs and environmental toxins, microsomes, RNA and DNA were isolated from human liver tissues. The genotyping of human liver samples was assessed by allelic discrimination. The amount of CYP proteins and transcripts were determined using western blot analysis and Real Time PCR (TaqMan assay) analysis, respectively. The microsomes were then incubated with the investigated known and presumed CYP3A substrates. The produce metabolites were identified and quantified using HPLC and LC-MS/MS analysis. Enzyme kinetic data analysis was finally used to determine enzyme kinetic parameters. More specific details about the materials and assays used during this investigation are described within this section.

3.1 Materials

3.1.1 Instruments

Instruments	Manufacturer
HPLC pump L-7100	Merck, Darmstadt, Germany
HPLC autosampler L-7200	Merck, Darmstadt, Germany
HPLC ultraviolet detector L-7400	Merck, Darmstadt, Germany
LC-MS/MS API 2000	Applied Biosystems, Foster city, USA
Biophotometer (Instrument designed for analysis of DNA, RNA, Oligonucleotides and Proteins)	Eppendorf, Hamburg, Germany
Centrifuges	Kendro Laboratory Products, Germany Beckman, Munich, Germany Eppendorf, Hamburg, Germany
Sequence detection system ABI PRISM 7900HT (Real-Time Quantitative PCR System)	Applied Biosystems, Foster city, USA
Biorobot (Instrument designed for automating DNA isolation using a solid phase extraction method)	Qiagen, Hilden, Germany
Tecan Ultra (Microtiter plate - Fluorimeter)	Tecan, Crailshaim, Germany
p^H Meter	Knick, Berlin, Germany
Water bath (for microsomal incubation)	Kötterman, Uetze, Germany

3.1.2 Consumable materials

Products	Manufacturer
Adhesive PCR film	ABgene, Hamburg, Germany
Adhesive PCR foil seals	ABgene, Hamburg, Germany
Cuvette	Eppendorf, Hamburg, Germany
Tubes 15 ml	Greiner, Flacht, Germany
Tubes 50 ml	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Tubes 1.5 ml with cap	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
HPLC caps GL45	Merck, Darmstadt, Germany
Reaction tubes	Eppendorf, Hamburg, Germany
Flat Cap Strips	ABgene, Hamburg, Germany
Nitrile examination gloves	Ansell, München, Germany
Latex gloves	Kimberley-Clark, Koblenz, Germany
Cytostatic Gloves	Berner International, Elmshorn, Germany
Deuterium bulb for HPLC photometric detector	Merck, Darmstadt, Germany
Lichrospher 100 CN column (particle size, 5 µm; 125 X 4.6 mm)	Merck, Darmstadt, Germany
Nucleosil C18 column (particle size, 5 µm; 250 x 2.1 mm)	MZ-Analysentechnik, Mainz, Germany
Econosphere C18 column (particle size, 5µm; 4.6 x 250 mm)	Alltech, Unterhaching, Germany
Extraction C18 catridge (4.0 x 3.0 mm)	Phenomenex, Aschaffenburg, Germany
Optical adhesive covers	Applied Biosystems, Foster city, USA
Pipettes	Eppendorf, Hamburg, Germany
Pipettes with filter	Sarstedt, Nürmbrecht-Rommelsdorf, Germany
Microtiter optical sterile plate 96-well	Applied Biosystems, Foster city, USA
Protective clothing	Berner International, Elmshorn, Germany
Polyvinylidene fluoride membranes	Roche Diagnostics, Mannheim, Germany
Tank Blot Cell for Western blot	Biorad, Hercules, CA, USA

3.1.3 Chemicals

Chemicals	Manufacturer
Ampli Taq^{TM} DNA polymerase	Applied Biosystems, Foster city, USA
Ammonium acetate pro analysi	Merck, Darmstadt, Germany
Dexoynucleotide triphosphates (dNTP set)	ABgene, Hamburg, Germany
Taq DNA polymerase	Qiagen, Hilden, Germany
Random Primer pd (N) 6	Roche, Mannheim, Germany
TaqMan universal PCR master mix	Applied Biosystems, Foster city, USA
Tris base	AppliChem, Darmstadt, Germany
Glycine zur Analyse	AppliChem, Darmstadt, Germany
Sodium Dodecyl Sulfate	AppliChem, Darmstadt, Germany
N, N, N', N'- Tetramethylethylenediamine	AppliChem, Darmstadt, Germany
Ammonium persulfat zur Analyse	AppliChem, Darmstadt, Germany
EDTA	Merck, Darmstadt, Germany
Glycerine	Roth, Karlsruhe, Germany
Bromphenol blue	Roth, Karlsruhe, Germany
Ponceau S	AppliChem, Darmstadt, Germany
Pefabloc SC (protease inhibitor, prevents non-specific covalent modification of proteins)	Roche, Mannheim, Germany
Sucrose	Merck, Darmstadt, Germany
DTT	Sigma, Deisenhofen, Germany

ß-Mercaptoethanol	AppliChem, Darmstadt, Germany
NaCl	AppliChem, Darmstadt, Germany
Tween-20	AppliChem, Darmstadt, Germany
DL-Norleucin	AppliChem, Darmstadt, Germany
DNA standard 1 kb	Rapidozym, Berlin, Germany
Testosterone	Steraloids, Wilton, USA
6ß-hydroxytestosterone	Sigma, Deisenhofen, Germany
NADPH	Roche, Mannheim, Germany
Protein standard (BSA)	Biorad, Hercules (CA, USA)
Tacrolimus	Fujisawa, Osaka, Japan
13,15-O-didemethyltacrolimus	Dade Behring, Liederbach, Germany
13,31-O-didemethyltacrolimus	Dade Behring, Liederbach, Germany
13-O-demethyltacrolimus	Prof. Uwe Christians, University of Colorado Health Sciences Center, Denver, USA
15-O-demethyltacrolimus	Prof. Uwe Christians, University of Colorado Health Sciences Center, Denver, USA
31-O-demethyltacrolimus	Prof. Uwe Christians, University of Colorado Health Sciences Center, Denver, USA
Aflatoxin B1	Sigma, Deisenhofen, Germany
Aflatoxin Q1	Sigma, Deisenhofen, Germany
Aflatoxin M1	Sigma, Deisenhofen, Germany
Aflatoxin G1	Sigma, Deisenhofen, Germany
AFB1-GSH-conjugate	PD. Dr. A. Seidel, Biochemical Institute of Environmental Carcinogens, Grosshansdorf, Germany
Reduced glutathione (GSH)	Sigma, Deisenhofen, Germany
di-sodium hydrogen phosphate	Merck, Darmstadt, Germany
SuperSignal WestDura Chemiluminescence Substrate	Pierce, St Augustin, Germany

3.1.4 Kits/Reagents

Reagents	Manufacturer
Bradford reagent	Biorad, München, Germany
PicoGreen ds DNA reagent	Mobitec, Goettingen, Germany
Protein Assay Dye Reagent	Biorad, Hercules, CA, USA
Super signal west pico kit	Pierce biotechnology, Rockford, USA
Qiagen Blood Kit	Qiagen, Hilden, Germany
Tissue DNA kit	Qiagen, Hilden, Germany
RNeasy kit for RNA isolation	Qiagen, Hilden, Germany

3.1.5 Solvents

Solvents	Manufacturer
Acetone pro analysi	Geyer, Nürnberg, Germany
Acetonitrile pro analysi	Geyer, Nürnberg, Germany
Methanol pro analysi	Geyer, Nürnberg, Germany
Ethanol pro analysi	Geyer, Nürnberg, Germany

3.1.6 Enzymes

Enzymes	Manufacturer
SP6, T7 RNA polymerase	Promega, Mannheim, Germany
RQ-DNase	Promega, Mannheim, Germany

3.1.7 Drug metabolizing enzymes

Materials	Type	Manufacturer
Liver samples	Collection during surgical interventions	Department of Surgery, University Medical Center Charité, Humboldt University, Berlin, Germany
Single donor human liver microsomes	HGO3	Natutec, Frankfurt am Main, Germany
	HG64	Natutec, Frankfurt am Main, Germany
	HG74	Natutec, Frankfurt am Main, Germany
	HH31	Natutec, Frankfurt am Main, Germany
	HH54	Natutec, Frankfurt am Main, Germany
	HH47	Natutec, Frankfurt am Main, Germany
	HH91	Natutec, Frankfurt am Main, Germany
	HG95	Natutec, Frankfurt am Main, Germany
	HH108	Natutec, Frankfurt am Main, Germany
	HH86	Natutec, Frankfurt am Main, Germany
	HH3	Natutec, Frankfurt am Main, Germany
	HH1	Natutec, Frankfurt am Main, Germany
	HH89	Natutec, Frankfurt am Main, Germany
	HH48	Natutec, Frankfurt am Main, Germany
	HH9	Natutec, Frankfurt am Main, Germany
Pooled human liver microsomes	HLM	Natutec, Frankfurt am Main, Germany
Baculovirus-derived microsomes	CYP1A1/OR	Natutec, Frankfurt am Main, Germany
	CYP1A2/OR	Natutec, Frankfurt am Main, Germany
	CYP2A6/OR	Natutec, Frankfurt am Main, Germany
	CYP2A6/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP1B1/OR	Natutec, Frankfurt am Main, Germany

	CYP2B6/OR	Natutec, Frankfurt am Main, Germany
	CYP2B6/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP2C8/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP2C9*1/OR	Natutec, Frankfurt am Main, Germany
	CYP2C9*1/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP2C9*3/OR	Natutec, Frankfurt am Main, Germany
	CYP2C19/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP3A4/OR/b5	Natutec, Frankfurt am Main, Germany
	CYP3A4/OR	Natutec, Frankfurt am Main, Germany
	CYP3A5/OR	Natutec, Frankfurt am Main, Germany
	CYP3A7/OR/b5	Natutec, Frankfurt am Main, Germany
	OR/b5	Natutec, Frankfurt am Main, Germany
Cytochrome b5	Purified	Panvera, Karlsruhe, Germany
NADPH-Oxidoreductase	Purified	Panvera, Karlsruhe, Germany
Rat glutathione S-transferase (GST)	Purified	Sigma, Deisenhofen, Germany

3.1.7 Oligonucleotides*

Oligonucleotide	Sequence
CYP1A1 PCR primers	Forward 5'-CAA GGA CCT GAA TGA GAA GTT CTA CAG-3' Reverse 5'-TGG GGT TCT CAT CCA GCT G-3'
CYP1A1 probe	FAM-TGA GAA GGG CAA CAT CCG GGA CAT-TAMRA
CYP3A4 PCR primers	Forward 5'-TCA GCC TGG TGC TCC TCT ATC TAT-3' Reverse 5'-AAG CCC TTA TGG TAG GAC AAA ATA TTT-3'
CYP3A4 probe (absolute quantification)	TCC AGG GCC CAC ACC TCT GCC T
CYP3A5 PCR primers	Forward 5'-TTG TTG GGA AAT GTT TTG TCC TAT C-3' Reverse 5'-ACA GGG AGT TGA CCT TCA TAC GTT-3'
CYP3A5 probe (absolute quantification)	TAT TTA TGA CTG TCA ACA GCC TAT GCT GGC TAT CA

* All oligonucleotides were purchased from Applied Biosystems, Foster City, USA

3.1.8 Antibodies

Antibodies	Type	Manufacturer
Anti-human CYP3A4/5, Cat. No. 458334	Monoclonal antibody, Immunoinhibition	Natutec, Frankfurt am Main, Germany
Anti-human CYP1A2, Cat. No. P2733	Monoclonal antibody, Western Blot	Panvera, Karlsruhe, Germany
Rabbit Anti-human CYP3A4, Cat. No. 458234	Polyclonal antibody, Western Blot	Natutec, Frankfurt am Main, Germany
Rabbit Anti-human CYP3A5, Cat. No. 458235	Polyclonal antibody, Western Blot	Natutec, Frankfurt am Main, Germany

3.2 Methods

3.2.1 Genotyping

3.2.1.1 Isolation of genomic DNA

Genomic DNA was isolated from blood or liver samples using Biorobot or Qiagen blood and tissue DNA isolation kits.

3.2.1.2 RNA Isolation and cDNA synthesis

Following homogenization, total RNA was isolated using RNeasy kit. cDNA from liver samples was synthesized from 1 µg of total RNA using random hexamer primers (0.1 A_{260} units), dNTPs (0.3 mM), and 50U of SuperScript reverse transcriptase in a total volume of 30 µl using the buffer and reaction conditions provided by Invitrogen. cDNA samples were diluted to a final concentration of 10 ng/µl of the total RNA taken for reverse transcription, aliquoted, and stored at – 80°C.

3.2.1.3 DNA and RNA quantification

DNA and RNA concentrations were determined photometrically at 260 nm using the Biophotometer 6313 according to the manufacturer's guideline.

*3.2.1.4 Allelic discrimination (determination of CYP3A5*3 SNP*)

CYP3A5 gene was amplified by real time polymerase chain reaction (RT-PCR) using primers 5'-TTGTTGGGAAATGTTTTGTCCTATC-3' (forward) and 5'-ACAGGGAGTTGACCTTCATACGT

T-3' (reverse). The presence of the SNP and of the wild-type sequence CYP3A5*1 was simultaneously detected by use of 2 TaqMan MGB probes (Applied Biosystems, Foster city, CA) with sequence TGTCTTTCAATATCTCTTC labelled with FAM fluorescence reporter dye and probe TGTCTTTCAGTATCTCTT labelled with VIC fluorescence reporter dye. Allelic discrimination was accomplished by real-time PCR amplification performed with the ABI PRISM 7900 sequence detection system (Applied Biosystems) with the following conditions: 50°C for 2 min, then 95°C for 10 min, followed by 35 cycles of amplification at 92°C for 15 s and 62°C for 1 min. Data were analyzed with the sequence detector software (version 2.1; Applied Biosystems).

3.2.2 TaqMan analysis (RT-PCR)

Plasmids containing CYP3A4 and CYP3A5 cDNAs were obtained by PCR amplification and subcloning of reverse-transcribed liver mRNA using gene-specific primer pairs. Following linearization, sense mRNAs were obtained by *in vitro* transcription using SP6 or T7 RNA polymerase. Plasmid DNA was removed through digestion with RQ-DNase, and transcribed RNA was purified by phenol/chloroform extraction and precipitation. RNA yield was determined photometrically, and the number of transcripts per microgram of *in vitro* RNA was calculated based on the length of the respective RNA. cDNAs species were then synthesized as described above and serial 10-fold dilutions from 106 to 100 copies were used as calibration curves.

mRNA Expression levels were quantified by real-time quantitative PCR using the Applied Biosystems Prism™ Sequence Detection System 7700 (SDS7700). Oligonucleotides and TaqMan probes were developed using Primer Express software (Applied Biosystems). TaqMan probes were labelled on 5' end with the reporter dye 6-carboxy-fluorescein and on 3' end with the quencher 6-carboxy-tetramethylrhodamin. Usually, cDNA derived from 10 ng of total RNA was used for one TaqMan reaction. In case of low copy numbers, the amount was increased to 40 ng. TaqMan PCR amplifications were performed using the Universal Mastermix from Applied Biosystems or the TaqMan reaction system from Eurogentec. The conditions consisted of 40 cycles of 15 s at 95°C and 1 min at 60°C, following an initial step of 10 min at 95°C. Transcript numbers were then calculated using linear regression analysis from their respective calibration curves. The expression level of 18S

rRNA was determined using commercially available, predeveloped TaqMan assays (Applied Biosystems). Normalized expression data were obtained by dividing CYP3A transcript numbers by those of 18S rRNA. All assays were performed in triplicates. Analyses of most samples were performed at least twice with independent reverse transcription and TaqMan analysis.

3.2.3 *In vitro* metabolism

3.2.3.1 Human liver samples

Liver samples used in the testosterone study were collected during surgical interventions conducted at the Department of Surgery, University Medical Center Charité, and Humboldt University in Berlin, Germany. The donors were white Europeans. The liver samples included nontumorous tissue surrounding primary liver tumors and metastases of various tumors or liver material surgically removed for other reasons. Medical histories were evaluated in respect of treatment with known CYP3A inducers. The study was approved by the local ethics committee and conducted in accordance with the declaration of Helsinki.

The liver samples and derived microsomes used in tacrolimus and aflatoxin B1 studies were obtained from Natutec and are described in more detail in Materials, section 3.1.7 (Drug metabolism enzymes).

3.2.3.2 Preparation of Human liver microsomes

The preparation of human liver microsomes (HLM) was carried out by subcellular fractionation as described elsewhere (Pearce et al., 1996). In summary, liver tissue in homogenization buffer (20 mM Tris pH 7.4 at 4°C, 5 mM sodium EDTA, 254 mM sucrose, 0.2 mM Pefabloc SC) was weighted in a tumbler, minced with scissors, homogenized subsequently with an Ultraturrax homogenizer at 9,500 rpm for 30 seconds twice and with a Potter-Elvehjem (Glass tube) tissue grinder at 1000 rpm for 30 min. The obtained homogenate was centrifuged by 3000 g for 30 minutes in an Eppendorf 5810R centrifuge. The supernatant was removed and centrifuged by 17000 g for 30 minutes at +2°C in a J2-21M/E Beckman centrifuge. Then the obtained supernatant was removed and centrifuged by 100000 g for 60 minutes at +4°C in a L8-70 M Beckman Ultracentrifuge. The resulting supernatant (the

cytosolic fraction) was removed and stored at -80°C for other research purposes. The resting pellet (human liver microsomes) was resuspended in microsomal buffer (20 mM Tris pH 7.4 at 4°C, 5 mM sodium EDTA and 254 mM sucrose) and homogenized with Potter-Elvehjem tissue grinder at 1000 rpm. The microsomes were then aliquoted and stored at -80°C.

3.2.3.3 Protein quantification

The protein quantification in microsomal preparations was assessed using the Bradford method (Bradford, 1976) which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. In summary, the test samples for blank, BSA standards and the protein samples to be tested (unknown samples) were generated.. The concentration of protein standards (BSA) ranged from 2 to 20 µg /µl. The dye reagent (1:5 diluted in deionised water) was added to the samples which were then allowed to incubate for 15 min. Thereafter, each sample was measured at 595 nm using a UV-visible spectrophotometer (Biophotometer). The absorbance of BSA standard was plotted as a linear function of its theoretical concentration ("y = mx + b", where y = absorbance at 595 nm and x = protein concentration). Using this equation, the protein concentration of each unknown protein samples was calculated based on their measured absorbance.

3.2.3.4 Western Blot analysis

3.2.3.4.1 CYP3A4/5

Protein expression of CYP3A4 and CYP3A5 in liver samples was determined by Western blot using CYP3A4- and CYP3A5-specific antibodies. To obtain total protein homogenate, powdered liver tissue was homogenized in 0.1 M Tris-Cl pH 7.4, 1 mM EDTA, 1 mM Pefabloc, 1 µg/ml leupeptin with a Potter Elvehjem homogenizer (glass/Teflon) for 2 min at 1000 rpm. Homogenates were then sonicated for 30 s at 12 W and stored at – 80°C. For western blotting 12.5 µg microsomal protein homogenate or 40 µg total protein homogenate were separated in a 10% SDS-polyacrylamide gel and transferred to

polyvinylidene fluoride membranes in a TankBlot Cell by electroblotting at 70 mA for 1.5 hour in 25 mM Tris/40 mM DL-norleucin/20% methanol transfer buffer. Following the transfer, the membranes were blocked for 60 min in 5% (w/v) skim milk, TBS, 0.1% (v/v) Tween-20 (blocking buffer). Incubation with isoform-specific primary antibodies was performed for 1 hour as follows: rabbit anti-CYP3A4 1:500 in blocking buffer, rabbit anti-CYP3A5 1:1000 in 1% (w/v) skim milk, TBS, 0.1% Tween-20. Secondary antibody (anti-rabbit IgG-peroxidase Fab-fragments, 1:20 000, Dianova) incubations were carried out in blocking buffer for 30 min. 3A4 and 3A5 bands were detected with SuperSignal WestDura chemiluminescence substrate (Pierce, St Augustin, Germany) and a digital CCD-luminescence camera (LAS-1000 luminescent image analyzer, Fuji). Signal quantification was performed with AIDA software (Raytest, Straubenhardt, Germany). Protein expression levels were calculated based on calibration curves obtained with microsomal protein from human lymphoblasts expressing recombinant CYP3A4, or of microsomal protein from baculovirus infected insect cells expressing recombinant CYP3A5. The calibration curves contained 50, 125, 250, 500, 1000 fmol of CYP3A4 or 12.5, 25, 50, 100, 200 fmol CYP3A5. The apoprotein content of CYP3A4 and CYP3A5 in these calibration curves was investigated by western blot using heterelogously expressed, purified CYP3A4 and CYP3A5 (see above) and two different polyclonal antibodies which recognize both CYP3A4 and CYP3A5.

3.2.3.4.2 CYP1A2

The quantification of CYP1A2 protein in human liver microsomes was carried out by immunoblotting analysis using antibody against human CYP1A2 (anti-P450 1A2 monoclonal antibody). Microsomal protein (0.005 to 0.05 pmol varying amounts of recombinant CYP1A2 standard and 5 µg of liver microsomal protein) was denatured for 3 min at 100°C in 125 mM Tris buffer (pH 6.8) containing 2 mM EDTA, 4% SDS, 20% glycerol, 2% ß-mercaptoethanol and 0.02% bromphenol blue. Recombinant CYP1A2 (BD Gentest) was used to generate calibration standard in concentrations ranging from 0.005 to 0.05 pmol/lane. Protein was separated on a 7.5% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (Roche Diagnostics, Mannheim, Germany) by electroblotting at 70 mA for 1 hour in 25 mM Tris/40 mM DL-norleucin/20% methanol transfer

buffer. The blots were blocked for 1 hour with 3% BSA in TBS-T (0.1% Tween-20, 15 mM sodium chloride, and 1 mM Tris; pH 7.4). The blots were then incubated for 1 hour with primary antibody (monoclonal antibody mouse anti-human P450 1A2 1:2500 in 1% BSA/TBS-T buffer), washed, incubated with anti-mouse IgG peroxidase conjugate (1:20000 in 1% BSA/TBS-T buffer) for 1 hour, and washed again. CYP1A2 protein was detected by means of a chemoluminescence reaction. Blots were exposed to film, developed, and quantified by use of Quantity One 4.2.1 software (Biorad). The calibration curve was linear within the range of 0.005 to 0.05 pmol.

3.2.3.5 In vitro incubation

3.2.3.5.1 Testosterone

250 µl of testosterone solution in methanol (ranging from 20 to 160 µM) were evaporated under a stream of nitrogen in Eppendorf tubes. Testosterone was then dissolved in 40 µl of 50 mM potassium phosphate buffer (pH 7.4). Baculovirus-expressed (BE) cytochrome P450 or HLM pre-diluted in the same phosphate buffer were added in a volume of 50 µl. The reaction (total volume 100 µl) was initiated by the addition of 10 µl of 20 mM NADPH, dissolved freshly in the same phosphate buffer, and allowed to proceed for 5-20 min at 37°C in opened Eppendorf tubes in a shaking water bath. In some experiments, the incubation mixture (final volume 100 µl) was supplemented with purified NADPH-oxidoreductase and/or purified cytochrome b5 following a previously described method (Evert et al., 1997). Specifically, after addition, the mixtures were vortexed and left to stand for 15 min at room temperature before initiation of metabolism experiments. Thus, the incubations consisted of (final concentrations given) 50 mM potassium phosphate buffer (pH 7.4), BE cytochrome P450 (4-20 pmol/100 µl) HLM (25-200 µg protein/100 µl), 2 mM NADPH and 5 µM to 400 µM testosterone in a final volume of 100 µl. The reactions were stopped by adding ice-cold methanol (100 µl). The resulting mixture was centrifuged at 13000 rpm for 5 min. 100 µl of the supernatant were used for HPLC analysis. The formation of 6ß-hydroxytestosterone was linear with time between 5 and 20 min and with the protein over the range 25 to 250 µg (HLM) and 4 to 20 pmol P450 (BE).

Table 4 *In vitro* microsomal incubation (example of experimental protocol)

	Testosterone final concentration in 100 µl incubation volume					
	5 µM	25 µM	50 µM	100 µM	200 µM	400 µM
Testosterone 50 µM in methanol (µl)	10	50	100	200		
Testosterone 160 µM in methanol (µl)					125	250
Methanol (µl)	240	200	150	50	125	
Evaporation/ N_2, 37°C (min)	5	5	5	5	5	5
Addition of phosphate buffer (µl)	40	40	40	40	40	40
Pre-incubation (min)	5	5	5	5	5	5
Addition of human liver microsomal solution (2 µg/µl) in phosphate buffer (µl)	50	50	50	50	50	50
Pre-incubation (min)	5	5	5	5	5	5
Addition of 20 mM NADPH (µl)	10	10	10	10	10	10
Incubation in opened Eppendorf tubes (min)	10	10	10	10	10	10
Addition of ice cold methanol (µl)	100	100	100	100	100	100
Centrifugation at 13000 rpm (min)	5	5	5	5	5	5

3.2.3.5.2 Tacrolimus

The *in vitro* microsomal incubation of tacrolimus was conducted as described in the above experimental protocol (Table 4). Precisely, stock solutions of tacrolimus ranging from 5 to 25µM were prepared in methanol. Samples were evaporated under N_2, dissolved in 100 mmol/L potassium phosphate buffer (pH 7.4), and supplemented with either HLM or baculovirus-expressed cytochrome P450. The final incubation volume was 100 µL. The end concentration of tacrolimus ranged from 0.5 to 12.5 µM. The reaction mixture with CYP3A4/OR or CYP3A5/OR was additionally supplemented with purified cytochrome b5 (P450: b5 ratio of 1:1) following a previously described method (Evert et al., 1997) prior to the start of the reaction with NADPH. Specifically, after addition, the mixtures were vortexed and left to stand for 15 min at room temperature before initiation of metabolism experiments. The reaction was initiated through addition of NADPH (final concentration 1 mM), allowed to proceed for 10 min, and stopped using 200 µl of a freshly prepared stop solution composed of 0.3 mol/L $ZnSO_4$/methanol (3:7 v/v) containing internal standard (ascomycin 25 µg/L). Following reaction termination, the incubation mixtures were centrifuged at 10000 g for 5 min. Supernatants were transferred into new tubes and 100 µl used for the LC-MS/MS analysis. The formation of 13-O-demethyltacrolimus was linear with time between 5 and 20 min and with protein over the range from 5

to 50 µg for HLM and from 1 to 8 pmol for baculovirus-expressed CYP3A. The end concentrations of tacrolimus ranged from 0 to 12.5 µmol/L. The substrate consumption did not exceed 15% during the incubation time (10 min).

3.2.3.5.3 Aflatoxin B1

Stock solutions of AFB1 (ranging from 50 to 500 µM) were prepared in methanol and were allowed to evaporate under N_2. The following components were added: 100 mmol/L potassium phosphate buffer (pH 7.4), 5 mmol/L reduced glutathione, purified rat liver glutathione S-transferase (0.2 mg/ml) and HLM or baculovirus-expressed cytochrome P450. The final incubation volume was 100 µL. The end concentration of AFB1 ranged from 25 to 500 µM. The reaction mixture with CYP3A4/OR or CYP3A5/OR was additionally supplemented with purified cytochrome b5 (P450: b5 ratio of 1:1) prior to the start of the reaction with NADPH, following a previously described method (Evert et al., 1997). Specifically, after addition, the mixtures were vortexed and left to stand for 15 min at room temperature before initiation of metabolism experiments The reaction was initiated through addition of NADPH (final concentration 1 mM), allowed to proceed for 30 min, and stopped by the addition of 100 µl ice-cold methanol containing 10 µM AFG1 as an internal standard. The incubation mixtures were then centrifuged at 10000 g for 5 min. Supernatants were transferred into new tubes and 100 µl were used for the HPLC analysis. The formation of AFQ1 and AFBO was linear with time between 10 and 30 min and with protein rangingfrom 100 to 500 µg for HLMs and from 20 to 100 pmol for baculovirus-expressed cytochromes. The substrate consumption was <15% over the incubation time (30 min).

3.2.3.6 Immunoinhibition

In immunoinhibition experiments, HLM or BE enzymes in phosphate buffer were supplemented with a monoclonal inhibitory antibody (MAB 3-29-9) against human P450 3A4/5 (Gelboin et al., 1995) at a ratio of 10 pmol P450 to 100 µg antibody, and incubated for 15 min on ice. Thereafter, the incubates were added to testosterone (final concentration 200 µM), dissolved in potassium phosphate buffer, and the activity measurements carried out and terminated as described above.

3.2.4 HPLC analysis

3.2.4.1 Testosterone

Following reaction termination, the incubation mixtures were centrifuged at 13000 rpm for 5 min. Supernatants were transferred into new tubes and 100 µl used for HPLC analysis. The HPLC system consisted of a L-7100 pump (Merck, Germany) and a Lichrospher 100 CN column (particles of silica with γ-cyanopropyl function, particle size, 5 µm; 100 Å, 125 X 4.6 mm ID, Merck, Germany). The mobile phase consisted of 78% (v/v) of 50 mM sodium phosphate buffer (pH 6.0), 16.5% (v/v) methanol and 5.5% (v/v) acetonitrile. The flow rate was 0.8 ml/min at room temperature. The absorbance was measured at 242 nm with an ultraviolet (UV) detector (L-7400 Lachrom, Merck, Germany). The retention times of 6ß-hydroxytestosterone and testosterone were 5.35 and 15.05 minutes, respectively.

3.2.4.2 Aflatoxin B1

The HPLC system consisted of a L-7100 pump (Merck, Germany) and an Alltech Econosphere C18 column (particles of silica with octadecyl derivative, particle size, 5µm, 80 Å 4.6 x 250 mm, Alltech, Deerfield, IL 60015). Peaks were eluted using a gradient program with solvent A (75% 50 mM sodium phosphate buffer (pH 3.0) with 25% methanol) and solvent B (methanol). Flow rate was 1 ml/min. The solvent gradient began with 90% A, 10% B, reaching 65% A, 35% B at 15 min, and 49% A, 51% B at 20 min, after which it was returned to 75% A, 25% B over 5 min, then held for 10 min. The absorbance was measured at 365 nm with an ultraviolet detector (L-7400 Lachrom, Merck, Germany). The retention times of AFB1-8,9-epoxide-GSH conjugate, AFQ1, AFM1, AFG1 and AFB1 were 9.15, 14.05, 16.3, 19.85 and 21.60 minutes, respectively. This method does not separate the AFB1-8,9-endo- and –exo-epoxide stereoisomers. The standard curves were linear from 50 to 20000 pmol for AFBO and from 25 to 20000 pmol for AFB1.

3.2.5 LC-MS/MS analysis

3.2.5.1 Tacrolimus

An online-extraction method with a column switching technique combined with analytical liquid chromatography and electrospray-tandem-mass-spectrometry was used to quantify 13-O-demethyltacrolimus. The supernatants obtained from microsomal incubations were injected with a series 200 auto-injector (Perkin Elmer). The extraction cartridge used was a security guard C18 4.0 x 3.0 mm (Phenomenex) and the analytical column was a Nucleosil 250 x 2.1 mm, 5 µm, 100 Å, C18 reversed phase column (particles of silica with octadecyl derivative, MZ-Analysentechnik) maintained at 65°C with a Du Pont column oven. The sample was introduced onto the extraction column at a flow rate of 1500 µl/min with deionized water (solvent A) for 0.5 min. After switching the diverter valve, analytes were eluted at a flow rate of 500 µL/min onto the analytical column with the following stepwise elution protocol: 0.5 to 3 min 65% solvent B (0.01g/L formic acid/2 mmol/L ammonium acetate in methanol), 3 to 4 min 75% B, 4 to 6 min 80% B, 6 to 9.5 min 100% B. The extraction column was then re-equilibrated with solvent A for 0.5 min at 1500 µL/min before starting the next injection. An API 2000 triple stage quadrupole instrument was used for quantification in the positive ion mode. Multiple reaction monitoring (MRM) with argon as collision gas and the following mass-transitions (ammonium adducts) were used for quantification: 13-O-demethyltacrolimus m/z 807.5/754.5, ascomycin m/z 809.5/756.5. Peak areas for 13-O-demethyltacrolimus were linear from 0.5 to 5 µg/l.

3.3 Data analysis

3.3.1 Software

Table 5 Computer programs applied

Software	Application	Source
Excel 2000 (Microsoft Office)	Correlation studies Linear regression Lineweaver-Burk plot (K_m, V_{max})	Microsoft, Redmont, WA, USA
SigmaPlot 8.0	Nonlinear regression	Chicago, IL, USA
SPSS 12.0	Parametrical analysis Nonparametrical analysis	Chicago, IL, USA
Sequence Detection Software	Version 2.1	Applied Biosystems, Foster city, USA
HPLC Integration software	D-7000 HPLC-system manager	Merck, Darmstadt, Germany
AIDA software	Signal quantification	Raytest, Straubenhardt, Germany
Quantity One Software	Version 4.2.1	Biorad, Hercules (CA, USA)

Table 6 Electronic Databases

Database / Website	URL
Directory of P450-Containing Systems	http://www.icgeb.trieste.it
Cytochrome P450 Allele Nomenclature	http://www.imm.ki.se/cypalleles/
Drugs Metabolized by Cytochrome P450	http://www.georgetown.edu/departments/pharmacology/clinlist.html
Pubmed (NIH)	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

3.3.2 Enzyme kinetic data analysis

The enzyme kinetic parameters (K_m, V_{max}) were determined either by nonlinear regression analysis (SigmaPlot 8.0; SPSS) for the tacrolimus- and AFB1-projects and by linear regression analysis (Excel 2000 Microsoft, Redmond, WA, USA) for the testosterone-project based on the following equations (Segel, 1975):

$$V = \frac{V_{max} \cdot [S]}{K_m + [S]}$$

$$\frac{1}{V} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

3.3.3 Calculation of relative contributions of the individual P450s

The relative contribution of each P450 isoform (i) to the overall rate of product (6ß-hydroxytestosterone, 13-O-demethyltacrolimus, AFB1-8,9-epoxide) formation (f_i) was predicted as a function of substrate concentration (S), using the relative hepatic abundance (A_i) of each recombinant CYP3A, as determined by quantitative Western-blotting using isozyme-specific antibodies, and the reaction velocity $V_{i(S)}$ based on the recombinant enzyme kinetic parameters (K_m, V_{max}) determined for each enzyme (Venkatakrishnan et al., 2000):

$$f_i(\%) = \frac{A_i v_i(S)}{\sum_{i=1}^{n} A_i v_i(S)} \cdot 100$$

3.3.4 Prediction of pharmacokinetic clearance

The kinetic parameters of baculovirus-expressed isoforms were used to estimate the *in vitro* intrinsic clearance of tacrolimus to 13-O-demethyltacrolimus. The intrinsic clearance was calculated as the V_{max}/K_m ratio (Houston, 1994). The net *in vitro* human liver microsomal intrinsic clearance was calculated for each CYP isoform, weighted by their respective baculovirus relative activity factor estimates, as defined previously (Venkatakrishnan et al., 2001). This was then scaled up to *in vivo* intrinsic clearance using previously published values of scaling factors: 50 mg of microsomal protein per gram of liver, and 20 g liver per kilogram of body weight (Carlile et al., 1999). The resulting estimated intrinsic clearance of tacrolimus via 13-O-demethylation (CL'_{int}) was used in conjunction with estimates of human hepatic blood flow (Q, 20 mL/min/Kg) and the free fraction of tacrolimus in human plasma (f_u, 0.01) (Iwasaki et al., 1996) to predict intravenous clearance via 13-O-demethylation. This was done according to the well-stirred (CLp_1) and parallel-tube models (CLp_2) (Obach et al., 1997; Obach, 1999) and the respective following equations (Wilkinson and Shand, 1975; Pang and Rowland, 1977c; Pang and Rowland, 1977b; Pang and Rowland, 1977a):

$$CLp\_1 = \frac{Q \cdot f_u \cdot CL'_{int}}{Q + f_u \cdot CL'_{int}}$$

$$CLp\_2 = Q \cdot \left(1 - e^{\frac{-CL'\text{int} \cdot fu}{Q}} \right)$$

3.3.5 Statistical analysis

The significance of differences in values between different groups was assessed by the T-test. The significance of differences in predicted clearance values between different groups was assessed using the Mann-Whitney U Test. The association between two variables was assessed using the Spearman rank test in the case of nonparametric data or by the Pearson correlation coefficient (SPSS 12.0) in the case of Gaussian-distributed data. Multiple correlations were also calculated using Excel 2000 (Microsoft, Redmond, WA, USA).

3.4 Method validation

3.4.1 Incubation

3.4.1.1 Solubility

All investigated substances were dissolved in the buffer under the concentration ranges used. Control experiments detected >90% of the substrates (testosterone, tacrolimus and aflatoxin B1) in the buffer following incubation at 37°C.

3.4.2 HPLC and LC-MS/MS analysis

3.4.2.1 Limit of detection (limit of quantification)

The limit of detection, the smallest amount of unknown that can reliably be detected was calculated as the amount of analyte, which produced a response that was three times larger than the standard deviation of the instrumental noise level. The limit of quantification, the smallest amount of unknown that can be identified and quantitatively measured was calculated as the amount of analyte measured

with accuracy between 80% and 120%, and with a precision not exceeding 20%. The detection (quantification) limit was 20 (50) pmol and 15 (40) pmol of testosterone and 6ß-hydroxytestosterone, respectively. The limit of detection (quantification) was 25 (50) and 10 (25) pmol of AFBO and AFB1, respectively. The limit of detection (quantification) for tacrolimus and 13-O-demethyltacrolimus was 0.1 (0.25) µg/l.

3.4.2.2 Intra-day variability (inter-day variability)

The intra-assay coefficient (inter-assay coefficient) was determined by measuring the formation of the produced targeted metabolite in a series of *n* (ranging from 10 to 20) identical incubates on the same day (different days) using aliquots of the same HLM lot.

The intra-day coefficient of variation for the formation of 6ß-hydroxytestosterone was 12.4%. The inter-day coefficient of variation for the formation of 6ß-hydroxyestosterone was 11.8%. The intra-day coefficient of variation for the formation of AFBO was 5.09%. The inter-day coefficient formation of 13-O-demethyltacrolimus was 2.7%. The inter-day coefficient of variation for the formation of AFBO was 14.8%. The intra-day coefficient of variation for the formation of 13-O-demethyltacrolimus was 7%.

4 RESULTS

4.1 Determinants of CYP3A5 activity *in vitro*

It is still disputed whether CYP3A5 activity is similar to or lower than that of CYP3A4 under *in vitro* and *in vivo* conditions. The activities of CYP3A isozymes had been shown previously to be affected by several chemicals and proteins, but the optimal conditions and the existence of isozyme-specific differences among CYP3A are still a matter of debate. Therefore, we assessed to investigate the optimal conditions of the *in vitro* activity of CYP3A5 compared to that of CYP3A4 using testosterone as a model substrate.

Divalent cations such as Ca^{2+} and Mg^{2+} at concentrations ranging from 5 to 50 mM have been previously shown to affect the *in vitro* activity of CYP3A4 (Yamazaki et al., 1995; Maenpaa et al., 1998). In this work, 25 mM of either Ca^{2+} or Mg^{2+} were used to investigate their role on the activity of baculovirus-expressed (BE) CYP3A5/OR *in vitro* compared to that of CYP3A4/OR. The measurements were conducted at 200 µM testosterone, which is approximately double the Km value of this reaction catalyzed by CYP3A4 and CYP3A5. The results are depicted in figure 7. The addition of $CaCl_2$ to a final concentration of 25 mM reduced the activity of either BE CYP3A by 70-100%. On the other hand, 25 mM Mg^{2+} halved the activity of BE CYP3A4 and CYP3A5

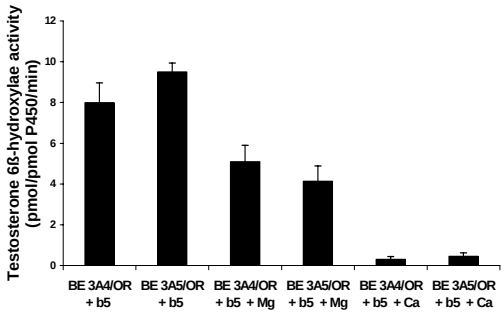


Fig. 7 Effect of Ca^{2+} and Mg^{2+} on the activities of baculovirus-expressed (BE) CYP3A4 and CYP3A5. The *y* axis represents the velocity of testosterone 6ß-hydroxylation at 200 µM testosterone. Slash (/) indicates co-expression (with OR) and "+" supplementation by addition of separately expressed protein (b5 as cytochrome b5). Each bar represents the mean of two experiments carried out in triplicate. Each error bar represents the standard deviation of experiments carried out in triplicate.

To determine whether the co-expressed OR in commercially available BE CYP3A/OR is sufficient or saturated, the effect of purified OR on the activity of BE CYP3A4/OR and CYP3A5/OR (slash indicates co-expression) were investigated. Only a concentration-dependent decrease of the rate of 6ß-hydroxytestosterone formation by either CYP enzyme was observed, indicating that the coexpressed preparations contained saturating amounts of OR (Fig. 8). The same BE CYP3A4/OR and CYP3A5/OR were used to investigate the effect of cytochrome b5, by adding increasing amounts of BE b5/OR (i.e. b5 co-expressed with OR). This gradually increased the testosterone hydroxylation activity of CYP3A4/OR until a plateau of activation (4-fold) was reached at a 10-fold excess of cytochrome b5/OR. The activity of CYP3A5/OR increased 3-fold upon addition of b5/OR and the maximum was seen at a ratio of CYP3A5/OR to b5/OR of 1:10 (Fig. 9). These increases were in all likelihood caused by b5 and not by the co-expressed OR, since, as described above, the addition of purified OR alone decreased the activity of CYP3A4/OR and of CYP3A5/OR. This is also supported by the effect of purified b5, which increased the activities of CYP3A4/OR or CYP3A5/OR 3-4-fold (Fig. 10). Whereas the magnitude of the effect was similar for purified b5 and OR/b5, purified b5 achieved its maximal effect at lower ratios to P450/OR than did OR/b5.

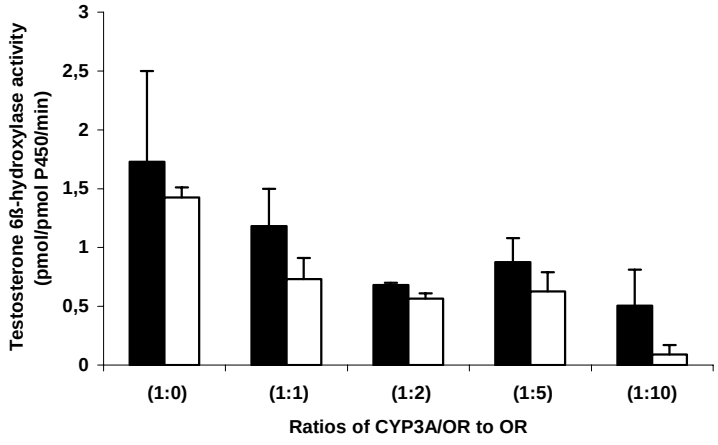


Fig. 8 Effect of OR ratio on the activities of baculovirus-expressed (BE) CYP3A4/OR (black bars) and of CYP3A5/OR (white bars). The *y* axis represents the velocity of testosterone 6ß-hydroxylation at 200 µM testosterone. Each bar represents the mean of two experiments carried out in duplicate. Each error bar represents the standard deviation of two experiments carried out in duplicate. The ratios of CYP3A/OR to OR are given in parentheses.

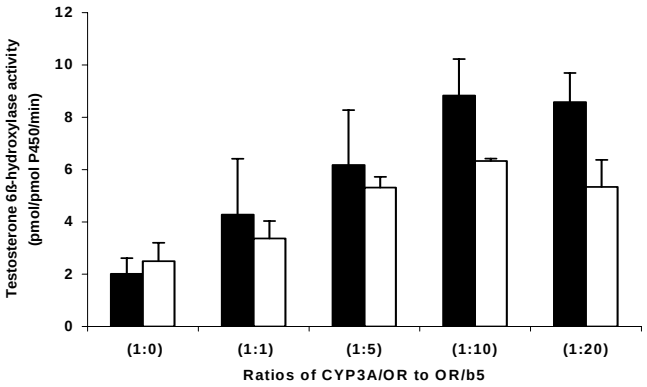


Fig. 9 Effect of OR/b5 on the activities of baculovirus-expressed (BE) CYP3A4/OR (black bars) and of CYP3A5/OR (white bars). The *y* axis represents the velocity of testosterone 6ß-hydroxylation at 200 µM testosterone. Each bar represents the mean of two experiments carried out in duplicate. Each error bar represents the standard deviation of two experiments carried out in duplicate. The ratios of the co-expressed CYP3A/OR to OR/b5 are given in parentheses

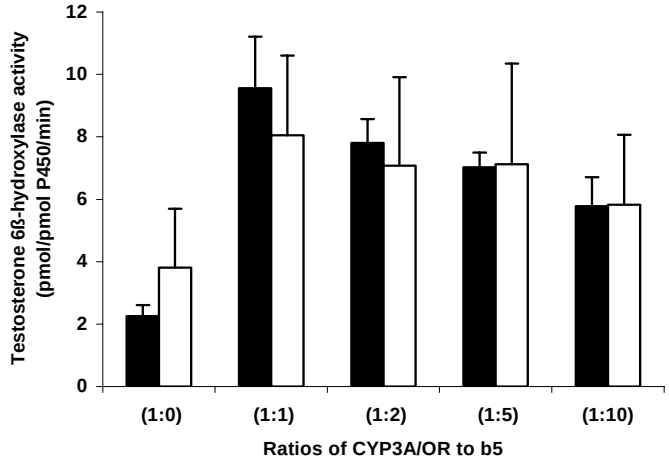


Fig. 10 Effect of b5 on the activities of baculovirus-expressed (BE) CYP3A4/OR (black bars) and CYP3A5/OR (white bars). The *y* axis represents the velocity of testosterone 6ß-hydroxylation at 200 µM testosterone. Each bar represents the mean of two experiments carried out in duplicate. Each error bar represents the standard deviation of two experiments carried out in duplicate. The ratios of the co-expressed CYP3A/OR to b5 are given in parentheses

In addition to b5 and OR, CYP3A4 and CYP3A5 activities could be influenced by some other, unknown proteins. To test for their presence, we supplemented CYP3A4/OR/b5 and CYP3A5/OR+b5 (i.e. CYP3A5/OR supplemented by b5 at a ratio of 1:1) by HLM. In either case, the activities of the respective P450 and HLM were additive. In contrast, there was a synergy between the activities of CYP3A5/OR and HLM. In all likelihood, this latter effect was caused by supplementation of b5 by HLM (Fig. 11).

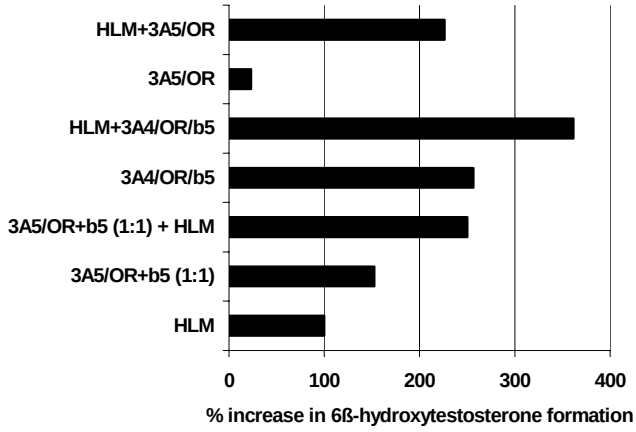


Fig. 11 Effect of the baculovirus-expressed (BE) CYP3A4 and CYP3A5 on 6ß hydroxytestosterone production by pooled human liver microsomes. Each bar represents the mean of two experiments carried out in duplicate. The testosterone concentration was set at 200 µM. The data are peak area of 6ß-hydroxytestosterone expressed in %, with that measured for pooled human liver microsomes (HLM) taken as 100%. The corresponding values for HLM, 3A5/OR +b5, HLM + 3A5/OR +b5, 3A4/OR/b5, HLM + 3A4/OR/b5, 3A5/OR and HLM + 3A5/OR are 100%, 152.5%, 250%, 256.5%, 361.5%, 23.30% and 226.5%, respectively. As shown in the figure, in contrast to others, only the addition of 3A5/OR to HLM resulted to a synergy (23.3% + 100% →226.5%), which is caused by the presence of cytochrome b5 in HLM.

Next we compared the specific activities of CYP3A4 and CYP3A5 under optimal conditions. In the absence of b5, the specific activities of CYP3A4 and CYP3A5 were nearly identical (1.71±0.25 vs. 1.63±0.14 pmol/pmol CYP/min) (Fig.12). The molar ratio of BE CYP3A4/OR and CYP3A5/OR to b5 was 1:1. Upon supplementation with b5, the activities of BE CYP3A4/OR and CYP3A5/OR supplemented by b5 were higher, but again nearly identical (7.52±0.35 vs. 7.00±1.48 pmol/pmol CYP/min) (Fig.12). The method of b5 supplementation was a major determinant of activity, as demonstrated for CYP3A4. The supplementation of b5 via co-expression with CYP3A4 and OR (CYP3A4/OR/b5) resulted in a much higher activity than the addition to CYP3A4/OR of an optimal amount of b5 (49.2±0.37 vs. 7.52±0.35 pmol/pmol CYP/min) (Fig.12).

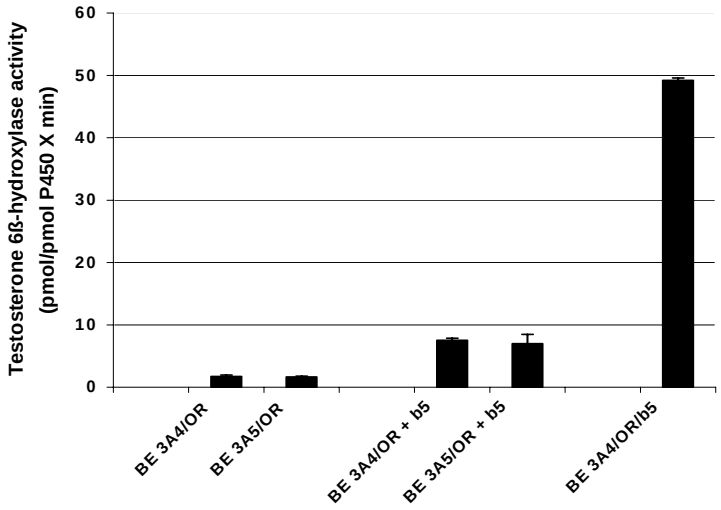


Fig. 12 Comparison of the testosterone 6ß-hydroxylase activity of CYP3A4 with that of CYP3A5 in the presence and absence of b5. Slash indicates co-expression and "+" supplementation by addition of separately expressed protein. The *y* axis represents the velocity of testosterone 6ß-hydroxylation at 200 µM testosterone. Each bar represents the mean of three experiments carried out in duplicate. Each error bar represents the standard deviation of three experiments carried out in duplicate. The ratio of BE CYP3A/OR to b5 was 1:1

4.2 Contribution of CYP3A5 to the hepatic 6ß-hydroxylation of testosterone

The specificity of 6ß testosterone hydroxylation as a marker of CYP3A was tested in microsomes from baculovirus cell lines expressing various individual CYP isozymes (Fig. 13). At 200 µM testosterone, the 6ß-hydroxylase activity of CYP1A1 was 1/3 of that of CYP3A4 and CYP3A5. All other CYPs tested showed low (CYP3A7, 0.72 pmol/pmol CYP/min; CYP1A2, 0.11 pmol/pmol CYP/min; CYP1B1, 0.57 pmol/pmol CYP/min; CYP2C19, 0.0265 pmol/pmol CYP/min) or no detectable 6ß testosterone hydroxylation activity (CYP2A6, CYP2B6, CYP2C9). The enzyme kinetic parameters (V_{max}, K_m) of testosterone 6ß-hydroxylation mediated by CYP3A4 and CYP3A5 are given in Table 7. Furthermore, we investigated the share of 6ß hydroxylation of testosterone catalyzed in HLM by CYP3A using an antibody, which inhibits selectively CYP3A4 and CYP3A5. A maximal inhibition of 85% was observed at a ratio of 10 pmol CYP to 100 µg antibody (Fig. 14).

Table 7 Enzyme kinetic parameters for testosterone 6ß-hydroxylation catalyzed by BE CYP3A4 and CYP3A5. The testosterone concentration range varied from 50 to 400 µM. The baculovirus-expressed enzymes were either co-expressed with b5 or supplemented with cytochrome b5. Each value represents the mean of two experiments carried out in duplicate. The ratio of BE CYP3A/OR to b5 was 1:1.

Baculovirus-Expressed Enzyme Systems	Enzyme kinetic parameters	
	K_m (µM)	V_{max} (pmol/pmol CYP/min)
CYP3A4/OR/b5	42.94	35.25
CYP3A5/OR	170.5	2.67
CYP3A4/OR + b5	128.8	14.5
CYP3A5/OR + b5	113.3	13.1

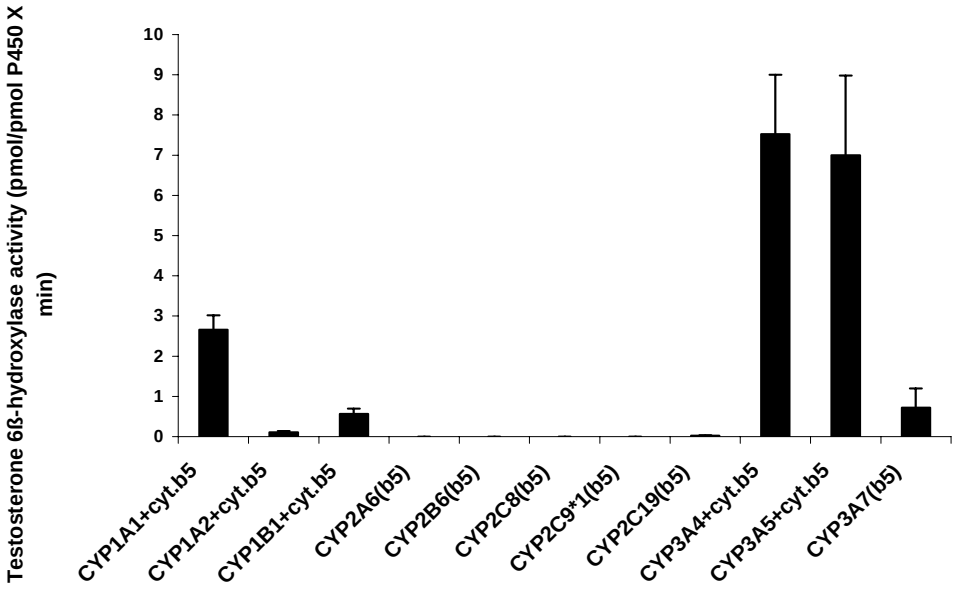


Fig. 13 Rate of 6ß-hydroxylation formation by baculovirus-expressed (BE) CYP3A4 and CYP3A5. The testosterone concentration was set at 200 µM. Each bar represents the mean of two experiments carried out in duplicate. Each error bar represents the standard deviation of two experiments carried out in duplicate All enzymes were either co-expressed with cytochrome b5 (b5) or were supplemented with b5 (+cyt.b5). The corresponding ratios of P450/OR to b5 were 1:1.

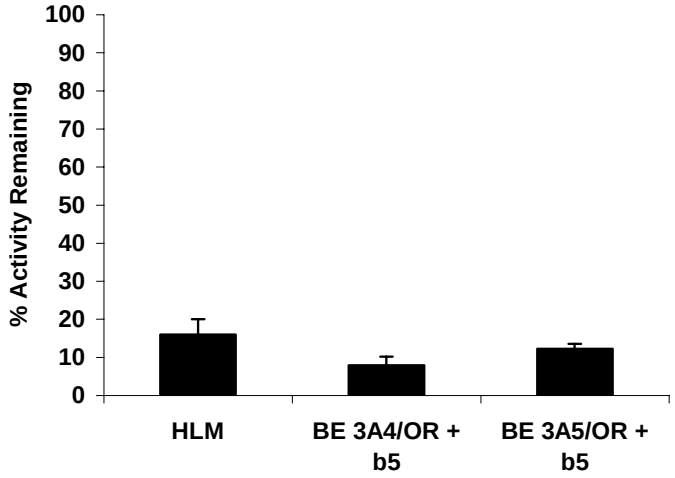


Fig. 14 Immunoinhibition of 6ß-hydroxytestosterone formation by HLM and Baculovirus-Expressed (BE) CYP3A4 and CYP3A5. Testosterone concentration was set at 200 µM. Each bar represents the mean of two experiments carried out in duplicate. Each error bar represents the standard deviation of two experiments carried out in duplicate. CYP3A4 and CYP3A5 were supplemented with b5. The ratio of BE CYP/OR to b5 was 1:1.

The variability of 6ß hydroxylation of testosterone was investigated in a bank of 47 liver samples obtained from Caucasians. The *in vitro* kinetic parameters of the reaction were determined at 50 µM, 200 µM and 400 µM testosterone concentrations. There was a 28-fold variability in the V_{max} values, which ranged from 346 to 9780 pmol/mg/min (median value 3336 pmol/mg/ min) (Table 8). The V_{max} and K_m mean values were 3387 pmol /mg/min and 90 µM, respectively. The V_{max}/K_m values varied 38-fold.

Table 8 Summary statistics of 6ß-hydroxylation of testosterone and of the expression of CYP3A proteins and of CYP3A and CYP1A1 mRNAs in 47 Caucasian livers.

Liver Samples	Summary Statistics	V_{max}	K_m	V_{max} / K_m	CYP3A5 protein	CYP3A4 protein	CYP3A4 mRNA	CYP3A5 mRNA	CYP3A7 mRNA	CYP3A43 mRNA	CYP1A1 mRNA
		pmol/mg/min	µM	µl/min/mg	pmol/mg		x 1000 copies/ng of total RNA				
3A5 *1/*3 Livers n = 6	Mean ± SD	2976 ± 2787	92 ± 43	29 ± 20	13 ± 3	64 ± 75	391 ± 118	36 ± 18	11 ± 11	1 ± 0.39	0.77 ± 0.61
	Median values	2406	104	38.5	14.8	20.7	407	25	10	0.88	0.77
	Range	346 –6220	34 – 138	3 – 51	9-16	11.5 – 188	248 – 526	21– 62	1.8 – 30	0.72 – 1.67	0.009 – 0.15
	Variability (fold)	18	4	15	1.78	16	2	3	17	2.3	162
3A5 *3/*3 Livers n = 41	Mean ± SD	3798 ± 2412	87.5 ± 36	45 ± 25	0	81 ± 77	451 ± 270	6 ± 8300	10 ± 15	1.38 ± 1.24	0.47 ± 0.53
	Median values	3466	86	37	0	50.7	380	4.26	2.9	0.79	0.29
	Range	400 –9781	14 – 164	5.4 – 115	0	4 – 375	695 – 170	2 – 10.5	0.18 – 56	0.14 – 5	0.007 – 2
	Variability (fold)	25	12	21	0	94	17	5	313	28	305

The V_{max} values were compared with the expression of the CYP3A and CYP1A1 genes, the protein products of which were found above to contribute significantly to 6ß hydroxylation of testosterone. The variabilities in the expression of CYP3A mRNA species and of the CYP3A4 and CYP3A5 proteins and the respective average values for the 47 livers investigated here are given in Table 8. The expression of CYP1A1 mRNA was low and varied 305-fold in the sample set (Table 8). The only significant correlations were found for CYP3A4 mRNA ($r=0.51$, $p<0.001$) and CYP3A4 protein ($r=0.66$, $p<0.001$) (Table 9, Fig. 15A, C). The correlation between V_{max} and CYP3A5 mRNA in the six livers from CYP3A5*1 heterozygotes was higher than that for CYP3A5*3 homozygotes (0.21 vs. 0.02), but it was not significant (Table 9, Fig.15B). The correlation between CYP3A5 protein and V_{max} in CYP3A5*1/*3 livers was 0.22 (Table 9, Fig.15D). The corresponding value for CYP3A5*3/*3 livers could not be calculated, because the protein expression was below the limit of quantification. Combining CYP3A4 mRNA expression with the expression of other CYP3A mRNA species did not increase the degree of correlation (Table 9).

Table 9 Correlations between V_{max} of 6ß-hydroxylation of testosterone and expression of selected P450 mRNAs and protein species.

V_{max} versus	Correlation coefficient (r)	Number of samples	P values
CYP3A4 protein	**0.66**	47	< 0.001
CYP3A4 mRNA	**0.51**	47	< 0.001
CYP3A5 mRNA: all livers	0.064	47	0.33
CYP3A5 mRNA: *1/*3 livers	0.21	6	0.34
CYP3A5mRNA: *3/*3 livers	0.02	41	0.45
CYP3A5 protein: *1/*3 livers	0.22	6	0.64
CYP3A7 mRNA	0.12	47	0.21
CYP3A43 mRNA	0.25	47	0.044
CYP3A4 plus CYP3A5 mRNA	**0.50**	47	< 0.001
Total CYP3A mRNA	**0.50**	47	< 0.001
CYP1A1 mRNA	0.17	47	0.12

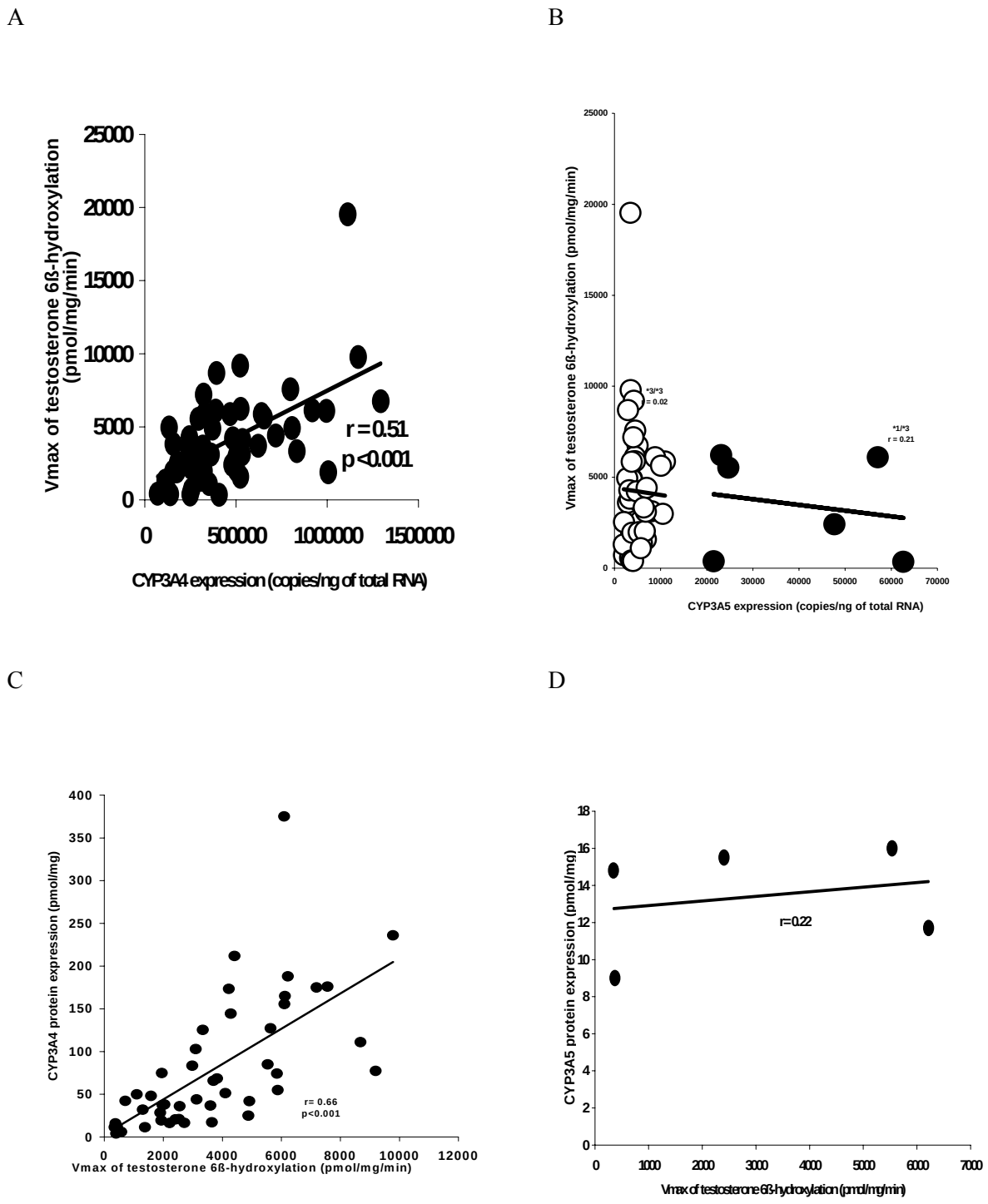


Fig. 15 Correlations between V_{max} of 6ß hydroxylation of testosterone and the expression of CYP3A4 mRNA (A), CYP3A5 mRNA (B), CYP3A4 protein (C), and CYP3A5 protein. (D)

The contribution of CYP3A5 to testosterone 6ß-hydroxylation was investigated in 5 CYP3A5*1/*3 livers using the relative hepatic abundance approach. At 200 µM testosterone, CYP3A5 accounted for 5%, 15%, 34%, 41% and 54% of the reaction in these livers (Table 10). The relatively high contribution of CYP3A5 in the latter three livers was caused by low expression of CYP3A4 (<21 pmol/mg protein).

Table 10. Relative contribution of CYP3A5 to testosterone 6ß-hydroxylation in 47 Caucasian liver samples.

selected HLM (n=47)	V_{max} (pmol/mg/min)	CYP3A4 (pmol/mg)	CYP3A5 (pmol/mg)	CYP3A4 contribution (%)	CYP3A5 contribution (%)
IKP148-006_3A5*1/*3	346.2	11.5	14.8	46.06	**53.94**
IKP148-007_3A5*1/*3	374.1	15.9	9	66	34
IKP148-041_3A5*1/*3	6220.1	188	11.7	94.64	**5.36**
IKP148-061_3A5*1/*3	2406.6	20.7	15.5	59.47	40.53
IKP148-085_3A5*1/*3	5536.7	85	16	85.38	14.62
IKP148-livers_3A5*3/*3 (n=42)	399.7-9780.7	4.1-375	N.D.	100	0

4.3 Contribution of CYP3A5 to the hepatic metabolism of tacrolimus

Previous investigations using human liver microsomes have indicated that CYP3A plays an important role in the metabolism of tacrolimus, the major primary metabolite being 13-O-demethyltacrolimus (Sattler et al., 1992; Karanam et al., 1994; Shiraga et al., 1994). Patients with high concentrations of CYP3A5 require higher doses of tacrolimus ((Macphee et al., 2002; Hesselink et al., 2003; Thervet et al., 2003; Zheng et al., 2003; Haufroid et al., 2004; Zheng et al., 2004; Macphee et al., 2005; Thervet et al., 2005). Better understanding of the role of CYP3A5 in tacrolimus disposition has been hampered by the paucity of *in vitro* data on tacrolimus metabolism by CYP3A5. Indeed, although tacrolimus metabolism to 13-O-demethyltacrolimus by CYP3A5 has been demonstrated (Bader et al., 2000), a detailed investigation on the kinetics of this reaction is lacking. Particularly informative would be a direct comparison with CYP3A4. Therefore, the relative contributions of CYP3A5 and CYP3A4 to the formation of the main tacrolimus metabolite (13-O-demethyltacrolimus) was set out to be assessed

using cDNA-expressed enzymes and a bank of human liver microsomes derived from low and high CYP3A5 expressors.

The specificity of 13-O-demethylation of tacrolimus was tested in microsomes from baculovirus cell lines expressing various individual CYP isozymes. At a concentration of 5 µM tacrolimus, CYP3A4 and CYP3A5 were the predominant CYP enzymes responsible for 13-O demethylation of tacrolimus and their corresponding catalytic activities were 0.54 and 0.79 pmol/CYP/min, respectively. CYP3A7 also exhibited some catalytic activity, but was 10-fold lower (0.065 pmol/pmol CYP/min). All other CYP tested showed extremely low 13-O demethylation activity (CYP1A2, 0.00058 pmol/pmol CYP/min; CYP2A6, 0.0014 pmol/pmol CYP/min; CYP1B1, 0.00084 pmol/pmol CYP/min; CYP2B6, 0.00064 pmol/pmol CYP/min; CYP2C8, 0.00084 pmol/pmol CYP/min; and CYP2C9, 0.00092 pmol/pmol CYP/min). Other metabolites, such as 13,15-O-demethyltacrolimus and 13,31-O-demethyltacrolimus were also detected, but their levels were 10-20 times lower than those of 13-0-demethyltacrolimus (Fig. 16). An example of a LC-MS/MS extracted ion peak chromatogram of 13-O-demethyltacrolimus, 15-O-demethyltacrolimus, 31-O-demethyltacrolimus, 13,31-O-didemethyltacrolimus and 13,15-O-didemethyltacrolimus is shown in Fig. 16

Panel A. **Panel B.**

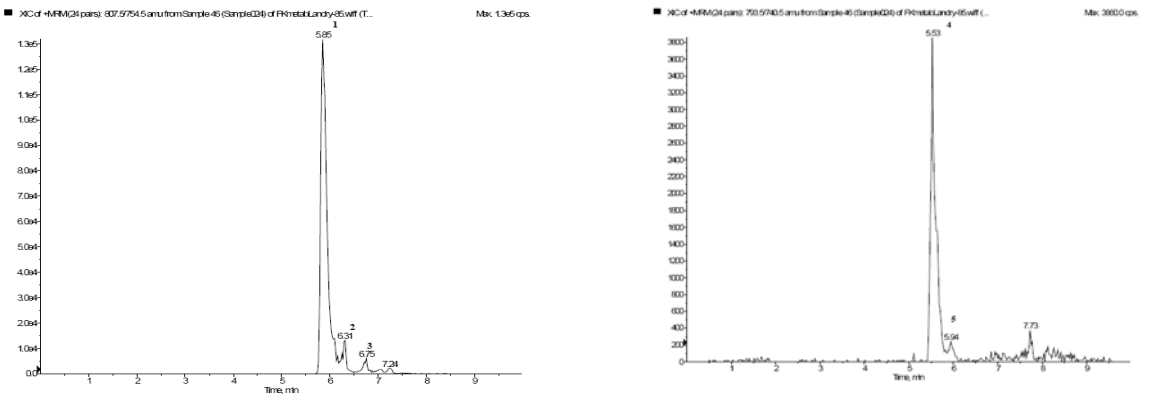


Fig. 16 LC-MS/MS extracted ion chromatograms of 100 µl supernatants of microsomal incubates of 13-O-demethyltacrolimus (1, Panel A), 15-O-demethyltacrolimus (2, Panel A), 31-O-demethyltacrolimus (3, Panel A), 13,31-O-didemethyltacrolimus (4, Panel B) and 13,15-O-didemethyltacrolimus (5, Panel B).

Next, the enzyme kinetic parameters for 13-O-demethylation of tacrolimus catalyzed by baculovirus-expressed CYP3A4 and CYP3A5 were determined. The tacrolimus concentration range varied from 0 to 12.5 µmol/L. The calculated K_m and V_{max} values using nonlinear regression analysis and fitting the data to the Michaelis-Menten equation were 1.5 µmol/L and 0.72 pmol/pmol P450/min for CYP3A4 (Fig. 17A), and 1.4 µmol/L and 1.1 pmol/pmol P450/min for CYP3A5 (Fig. 17B). The corresponding V_{max}/K_m ratios were 0.48 and 0.79 for CYP3A4 and CYP3A5, respectively.

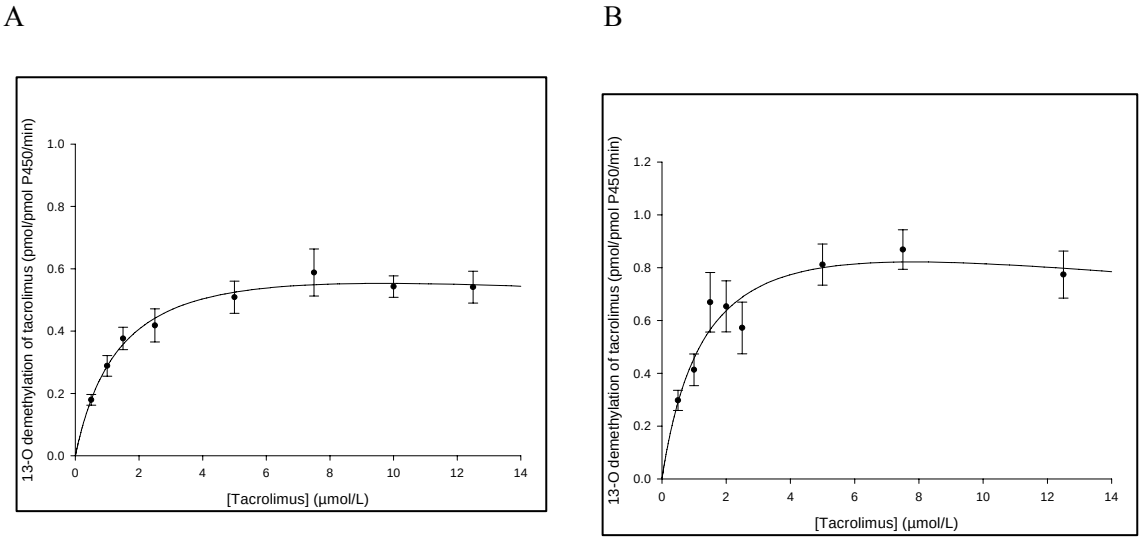


Fig. 17 Kinetic plots of 13-O-demethylation of tacrolimus by cDNA-expressed CYP3A4 (A) and CYP3A5 (B). CYP3A4 and CYP3A5 were supplemented with cytochrome b5. The ratio of CYP3A/OR to b5 was 1:1

Next, the tacrolimus 13-O-demethylation activity was investigated in a bank of 15 human liver samples, including 12 high expressors (homozygous or heterozygous carriers of CYP3A5*1) and 3 low expressors (homozygous carriers of CYP3A5*3) of CYP3A5. The relevant data are given in Table 11. There was an almost 19-fold variation in the V_{max} of tacrolimus 13-O demethylation with values ranging from 199.3 pmol /mg/min to 3746.7 pmol /mg x min (median: 564.8 pmol /mg/min) (Table 11). Less variation was observed in the respective K_m values (median: K_m 0.74 µmol/L; range 0.38 – 1.20 µmol/L).

Subsequently , we compared the V_{max} values obtained for the individual HLM with the corresponding protein contents of CYP3A4 and CYP3A5. The correlation coefficient (r) between V_{max} of tacrolimus 13-O demethylation and CYP3A5 protein was 0.65 (p=0.008) and was thus higher than that for V_{max}

and CYP3A4 protein 0.49 (p=0.09) (Fig. 18). We also observed a good correlation (r=0.64, (p=0.01), Fig. 18) between V_{max} values of tacrolimus 13-O demethylation and those of testosterone 6ß-hydroxylation (supplied in the package insert for each HLM). Since the latter reaction is a marker of the CYP3A activity (Kamdem et al., 2004), this result further confirms that tacrolimus is mostly metabolized by CYP3A. The data in Fig. 18 were also analyzed after the omission of the outlier with the particularly high V_{max} value. Significant correlations were still observed between V_{max} and either CYP3A5 protein (r=0.56, p=0.03) or testosterone 6ß-hydroxylation (r=0.57, p=0.03). No significant correlation was found between V_{max} and CYP3A4 protein (r=0.35, p=0.265) after exclusion of the outlier. No correlation was found between CYP3A4 and CYP3A5 protein in these samples (Fig. 18).

Table 11 Kinetic constants for the conversion of tacrolimus to 13-O-demethyl tacrolimus and estimated intrinsic tacrolimus clearances in a bank of human liver samples

Materials	V_{max} (pmol/mg/min)	K_m (µmol/L)	V_{max}/K_m (µl/mg/min)	CLp_1 (ml/min/kg)	CLp_2 (ml/min/kg)	CYP3A4 Protein[1] (pmol/mg)	CYP3A5 Protein[1] (pmol/mg)	Sex[1]	Age[1] (years)	T-6ß-OH Activity[1] (pmol/mg/min)
CYP3A5 LE livers (n=3)										
HG03	320.7	0.43	745.81	3.63	3.98	95	0.9	F	30	4600
HG64	199.3	0.51	390.78	2.43	2.58	NA	1.2	M	63	1800
HG74	336.4	1.2	280.33	3.78	4.16	NA	1.1	M	32	2600
CYP3A5 HE livers (n=12)										
HH54	671.3	0.56	1198.75	6.35	7.44	87	8.6	F	62	4300
HH31	3746.7	0.54	6938.33	14.44	18.51	300	14	F	51	12000
HH47	1129.5	0.47	2403.19	8.78	10.85	62	9.3	F	53	4100
HH91	1069.7	0.53	2018.3	8.51	10.46	46	5.7	F	55	2900
HG95	335.8	0.74	453.78	3.77	4.15	29	6.9	F	47	760
HH86	564.8	0.38	1486.32	5.62	6.47	83	7.6	M	57	3500
HH3	835	0.96	869.79	7.33	8.78	76	11	M	38	6500
HH108	448	0.86	520.93	4.74	5.33	27	11	F	27	3700
HH89	453.8	1.1	412.55	4.78	5.39	38	11	M	33	2200
HH48	457.7	0.85	538.47	4.81	5.43	30	9.8	M	62	1700
HH9	943.4	0.89	1060	7.9	9.59	53	12	M	51	5300
HH1	1122.9	1.1	1020.82	8.75	10.81	110	12	F	31	8000
Summary Statistics for all livers (n=15)										
Median	564.80	0.74	869.79	5.62	6.47	62.00	9.30		51	3700
Range	199.3 -3746.7	0.38 - 1.12	280.33 – 6938.33	2.43 – 14.4	2.58 – 18.51	27 – 300	0.9 – 14		27 – 63	760 – 12000
Summary Statistics for CYP3A5 HE livers (n=12)										
Median	753.15	0.79	1040.41	6.84	8.11	57.5	10.4		51	3900
Range	335.8 – 3746.7	0.38 –1.1	412.55 – 6938.33	3.77 –14.44	4.15 –18.51	27 - 300	5.7 - 14		27 - 62	760 – 12000

[1]data supplied by BD Gentest

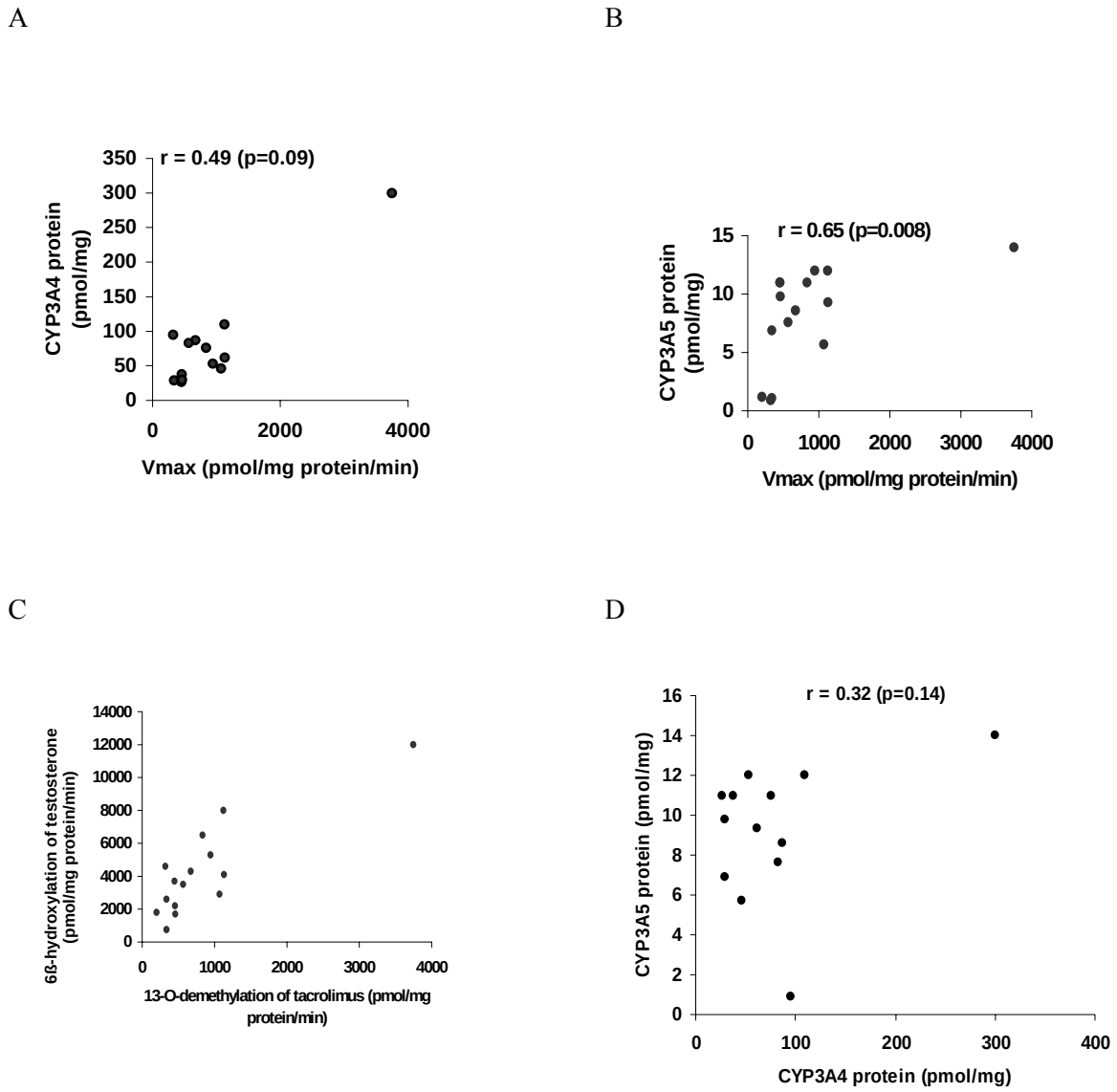


Fig. 18 Correlations between V_{max} of 13-O demethylation of tacrolimus and (A) CYP3A4 protein, (B) CYP3A5 protein, (C) 6ß-hydroxylation of testosterone. (D) Correlation between CYP3A4 protein and CYP3A5 protein.

The contribution of CYP3A5 to tacrolimus 13-O demethylation was determined in the liver bank using the relative hepatic abundance approach as described in Materials and Methods (section 3.3.3, calculation of relative contribution), assuming that the enzyme kinetic constants determined for the individual recombinant CYP3A isozymes were also valid for the hepatic liver microsomes. At 5 µmol/L tacrolimus, a concentration within the range measured in hepatic tissue *in vivo*, CYP3A5 accounted for 1.5% - 40 % of 13-O demethylation of tacrolimus. No significant association was found

between the contribution of CYP3A5 to 13-O-demethylation and the amount of CYP3A5 protein (Fig. 19A, r = 0.20, p = 0.51), but there was an inverse association with the amount of the CYP3A4 protein (Fig. 19B, r = 0.90, p<0.0001). Similar results were obtained at 0.5 and 50 µmol/L tacrolimus.

A B

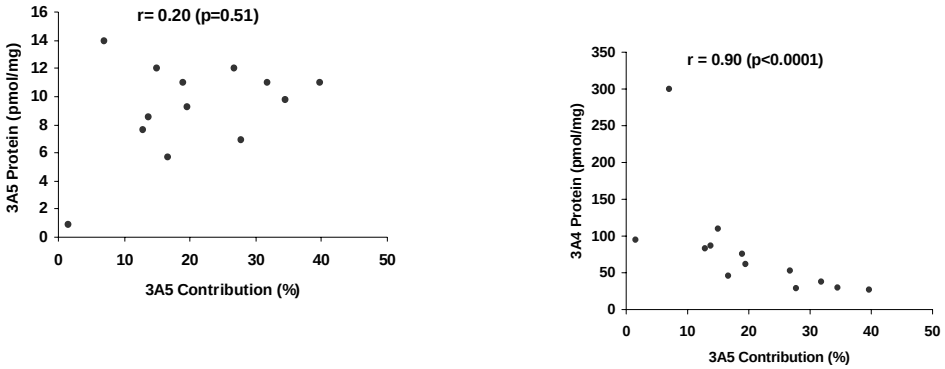


Fig. 19 Correlation between the relative contribution of CYP3A5 to the 13-O demethylation of tacrolimus and (A) CYP3A5 protein and (B) CYP3A4 protein in a liver bank (n=13, the 2 livers with no CYP3A4 protein data were excluded from the analysis). The relative contribution has been calculated based on the hepatic abundance approach as described in Materials and Methods. The tacrolimus concentration was 5 µmol/L.

Based on the relative activity factor approach (Venkatakrishnan et al., 2001) and using the parallel tube model , the predicted tacrolimus 13-O-demethylation clearance values (CLp2) in CYP3A5 high and low expressers livers were on average 8.60 ml/min/kg and 3.57 ml/min/kg of body weight, respectively (p=0.0088, Mann-Whitney U test) (Fig. 20). The predicted tacrolimus 13-O demethylation clearance values (CLp1) using the well-stirred model were also calculated and they were similar to the CLp2 values (Table 11).

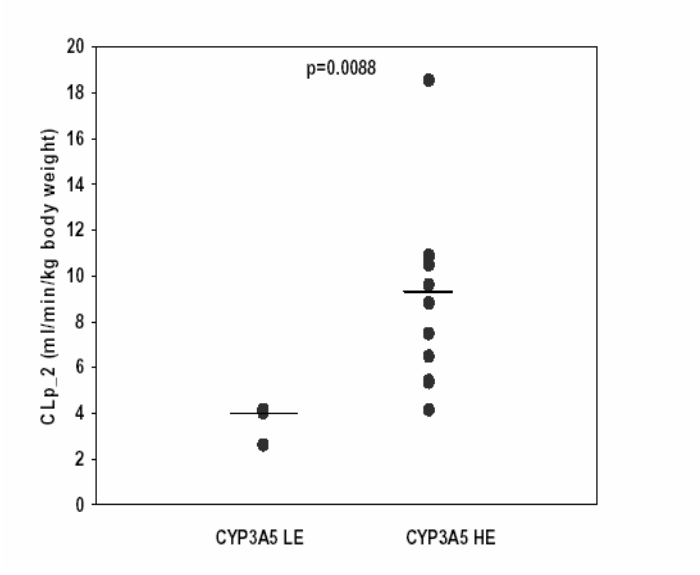


Fig. 20 Prediction of tacrolimus pharmacokinetic clearance of human liver samples using the parallel tube model as described in Materials and Methods. The horizontal line indicates the mean value of high (HE) and low CYP3A5 expressers (LE).

4.4 Contribution of CYP3A4, CYP3A5, CYP3A7 and CYP1A2 to the hepatic production of aflatoxin B1-8,9-epoxide

Previous investigations showed that CYP3A and CYP1A2 are the major enzymes involved in the metabolism of AFB1 in adult human liver microsomes (Forrester et al., 1990; Ramsdell et al., 1991; Ueng et al., 1995; Patterson et al., 1999). CYP3A4 produces predominantly AFB1-8,9-exo-epoxide and AFQ1, whilst CYP1A2 leads to the formation of AFB1-8,9-exo and endo-epoxide, and of AFM1. CYP3A5 metabolizes AFB1 mainly to the exo-epoxide (Wang et al., 1998), and so does CYP3A7 (Kitada et al., 1989; Kamataki et al., 1995; Yamada et al., 1998). However, there is still some discrepancy regarding the relative importance of CYP1A2 and CYP3A isoforms in human AFB1-8,9-exo-epoxide (the most carcinogenic and toxic AFB1 metabolite) formation. Therefore, the metabolism of AFB1 in a bank of liver samples phenotyped for the expression of CYP3A4, CYP3A5, and CYP1A2 was investigated. This was followed by a calculation of the relative contributions of the CYPs to AFBO formation in human livers at AFB1 concentrations encountered in exposed individuals.

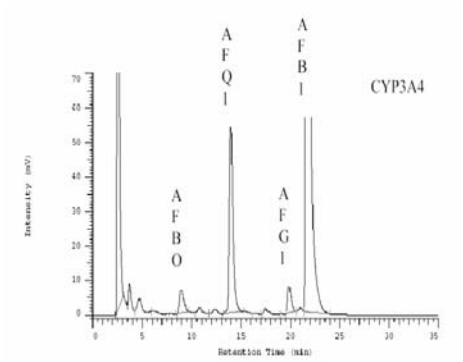
Representative HPLC chromatograms of AFB1 metabolism by baculovirus-expressed CYP3A5, CYP3A4, CYP3A7, and CYP1A2 are shown in Fig. 21 and the enzyme kinetic parameters describing the formation of the metabolites detected are given in Table 12. Within the AFB1 concentration range investigated (25 to 500 μM), CYP3A5 produced exclusively AFBO. CYP3A4 produced AFQ1 and AFBO (V_{max} ratio 8:1), but no AFM1. CYP3A7 had a similar profile of AFB1 metabolites as CYP3A4, with an AFQ1:AFBO V_{max} ratio of 23:1. In contrast to CYP3A4 and CYP3A7, CYP1A2 produced AFM1 and AFBO (V_{max} ratio 2.5:1), but no AFQ1. CYP1A2 had the lowest K_m values for AFM1 and AFBO formation. CYP3A4 showed the highest intrinsic clearance (V_{max}/K_m) towards AFBO , followed by CYP1A2, CYP3A5, and CYP3A7. CYP2A6, CYP2B6, CYP2C8 and CYP2C9 showed no detectable production of AFB1 metabolites.

Table 12 Kinetic constants for the conversion of AFB1 to AFQ1 and AFBO (AFB1-8,9-epoxide) by baculovirus-expressed CYP1A2, CYP3A4, CYP3A5, and CYP3A7. Each value represents the mean of two experiments carried out in duplicates.

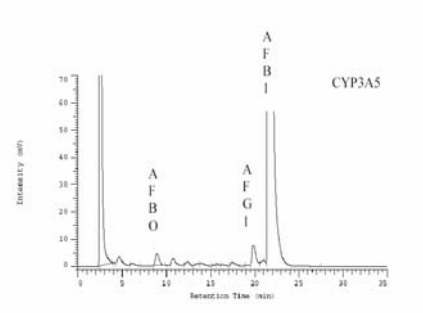
[a] **Kinetic constants**	**1A2/OR**	**3A4/OR+b5 (1:1)**	**3A5/OR+b5 (1:1)**	**3A7/OR/b5**
V_{max} AFBO	0.39	1.64	1.2	0.27
K_m AFBO	55	130	302	121
V_{max}/K_m AFBO	0.007	0.012	0.0039	0.0022
V_{max} AFQ1	N.D.	13.32	N.D.	6.35
K_m AFQ1	N.D.	324	N.D.	204
V_{max}/K_m AFQ1	N.D.	0.041	N.D.	0.031
V_{max} AFM1	0.95	N.D.	N.D.	N.D.
K_m AFM1	29	N.D.	N.D.	N.D.
V_{max}/K_m AFM1	0.032	N.D.	N.D.	N.D.

[a] pmol/pmol CYP/min (V_{max}), μM (K_m) and μl/pmol CYP/min (V_{max}/K_m). N.D., not detected.

A



B



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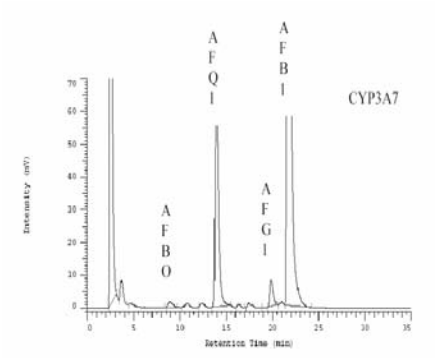

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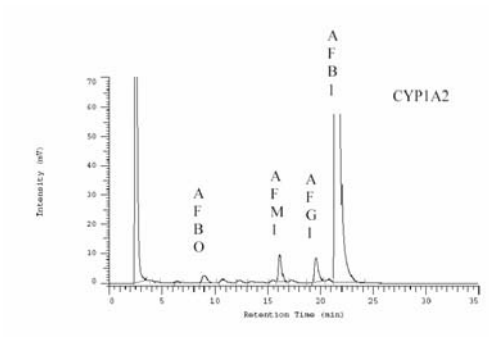


E

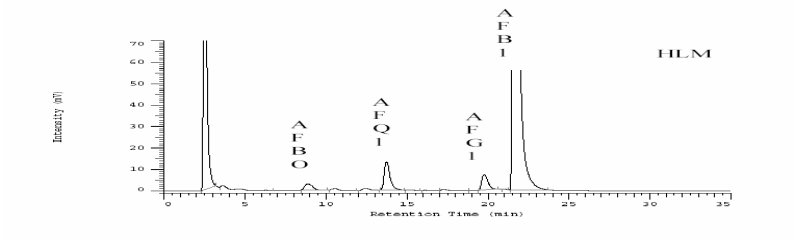


Fig. 21 Representative chromatograms of primary AFB1 metabolites formed by baculovirus-expressed CYP3A4 (A), CYP3A5 (B), CYP3A7 (C), CYP1A2 (D), and by human liver microsomes (HML, sample HH1 in Table 13) at 250 µM AFB1 (E). AFG1 was used as an internal standard. AFBO peak represents the glutathione conjugate of AFB1 8,9-epoxide.

AFB1 metabolism was then investigated in a set of human liver microsomes (HLMs) derived from 13 individuals. Protein expression data for the relevant P450s in these HLMs are given in Table 13. All but two donors (HG64 and HG74) were CYP3A5 high expressers. There was a 2-fold variability in the CYP3A5 expression among high expressers, whereas the corresponding value for all 13 livers was 11-fold. The variability in CYP3A4 expression was 4-fold and that of CYP1A2 was 16-fold. The correlation coefficient r between the expression of the CYP1A2 protein and the CYP1A2 activity marker phenacetin O-deethylase activity (Yuan et al., 2002) was 0.70 (p=0.003). CYP1A2 accounted on average for 2% of the total CYP content; the corresponding values for CYP3A4 and CYP3A5 were 20 and 3%, respectively. The correlation coefficients between the expression levels of the CYP3A4 protein versus CYP3A5 protein, CYP3A4 protein versus CYP1A2 protein, and CYP3A5 protein versus CYP1A2 protein were 0.18, 0.30, and 0.09, respectively (all 3 p values >0.05).

The major AFB1 metabolite detected in these HLM was AFQ1 followed by AFBO (Fig. 21, last panel, and Table 13). AFM1 was detected in none of the liver samples. There was a 12-fold variation in the V_{max} of the AFBO formation. The variation in V_{max} of AFQ1 formation was 22-fold. There was a statistically significant correlation between V_{max} values of AFBO and AFQ1 formation in the HLM set (Fig. 22). The ratio of AFQ1 and AFBO V_{max} median values in HLMs (7.2:1) was almost identical to the corresponding ratio obtained for CYP3A4 baculovirus-expressed enzyme (8:1) given above. We compared the kinetic parameters for the formation of these metabolites between the recombinant CYP3A4 and HLM. This was done with the consideration of the CYP3A4 expression levels in HLMs (Table 13). The median V_{max} (1.91 pmol/pmol P450/min), K_m (225 μM) and V_{max}/K_m (0.01 μl/pmol P450/min) values for AFBO formation as well as median V_{max} (13.74 pmol/pmol P450/min), K_m (415 μM) and V_{max}/K_m (0.033 μl/pmol P450/min) values for AFQ1 formation in this liver bank were very similar to the corresponding values measured with recombinant CYP3A4 (Table 12).

Table 13 Kinetic constants [a] for the conversion of AFB1 to AFBO and AFQ1 in a bank of microsomes derived from human liver samples

Materials	V_{max} (AFBO)	K_m (AFBO)	V_{max} (AFQ1)	K_m (AFQ1)	Total P450 content	3A4 Protein	3A5 Protein	1A2 Protein	Phenacetin O-deethylase activity (pmol/mg/min)	Sex	Age (years)
CYP3A5 LE (n=2)											
HG64	477	307	3560	312	340	NA	1.2	0.27	145	M	63
HG74	613	186	4308	450	250	NA	1.1	4.32	580	M	32
CYP3A5 HE (n=11)											
HH54	365	102	2865	283	240	87	8.6	3.72	230	F	62
HH47	840	414	11281	1762	260	62	9.3	2.34	360	F	53
HH91	332	106	6925	724	340	46	5.7	3.84	390	F	55
HG95	236	350	657	645	230	29	6.9	3.43	290	F	47
HH86	541	163	4046	310	330	83	7.6	2.40	300	M	57
HH3	967	267	1649	109	370	76	11	1.91	210	M	38
HH108	384	90	1587	112	310	27	11	3.76	330	F	27
HH89	261	125	2261	370	300	38	11	2.74	240	M	33
HH48	261	260	1334	256	210	30	9.8	1.80	130	M	62
HH9	782	249	8669	1098	210	53	12	4.27	660	M	51
HH1	4633	230	16871	1207	490	110	12	2.15	400	F	31
Summary Statistics for all livers (n=13)											
Mean	770	374	5552	685	299	58	8.24	2.84	328		47
Median	573	225	4124	415	300	53	9.3	2.74	300		42
Range	281-3366	104 -1553	753 - 16871	120-1937	210- 490	27 - 110	1.1 - 12	0.27-4.32	130 - 660		27 - 63
Summary Statistics for CYP3A5 HE (n=11)											
Mean	873	214	5286	625	299	58	9.53	2.94	321.81		46.90
Median	384	230	2865	371	300	53	9.8	2.74	300		51
Range	236-4632	90 - 414	657-16871	109-1762	210-490	27 - 110	5.7 - 12	1.8-4.27	130 - 660		27 - 62

[a] pmol/mg/min (V_{max}), μM (K_m) and pmol/mg (total P450, CYP3A4, CYP3A5, and CYP1A2).

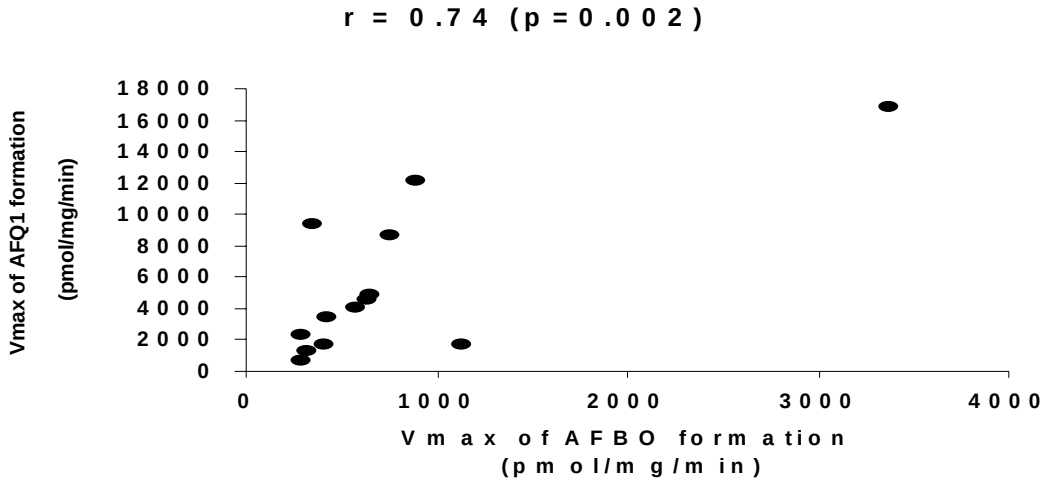


Fig. 22 Correlations between V_{max} values of AFBO and AFQ1 formations in a panel of human liver microsomes

These results suggested that most of AFQ1 and AFBO production was catalyzed by CYP3A4. To verify this, V_{max} values of AFQ1 and AFBO productions measured in the individual HLM were compared with the expression levels of CYP3A4, CYP1A2 and CYP3A5, and with the CYP1A2 activity marker phenacetin O-deethylase. In agreement with the dominant role of CYP3A4 in AFQ1 production, V_{max} of AFQ1 formation correlated statistically significantly with the expression of the CYP3A4 protein (Fig. 23A). The corresponding values for CYP3A5 (Fig. 23B) and CYP1A2 (both protein and phenacetin O-deethylase, Fig. 23C, D) were low and statistically insignificant, in agreement with the incapability of these P450s to catalyze AFQ1 (Fig. 21, Table 12). The correlation coefficient r was statistically significant between the CYP3A4 protein expression and V_{max} of AFBO formation (Fig. 23A). The corresponding values for CYP3A5 (Fig. 24B) and CYP1A2 (both protein and phenacetin O-deethylase, Fig. 24C, D) were lower and statistically insignificant, even though both of these P450s are capable of AFBO production (Fig. 21, Table 12).

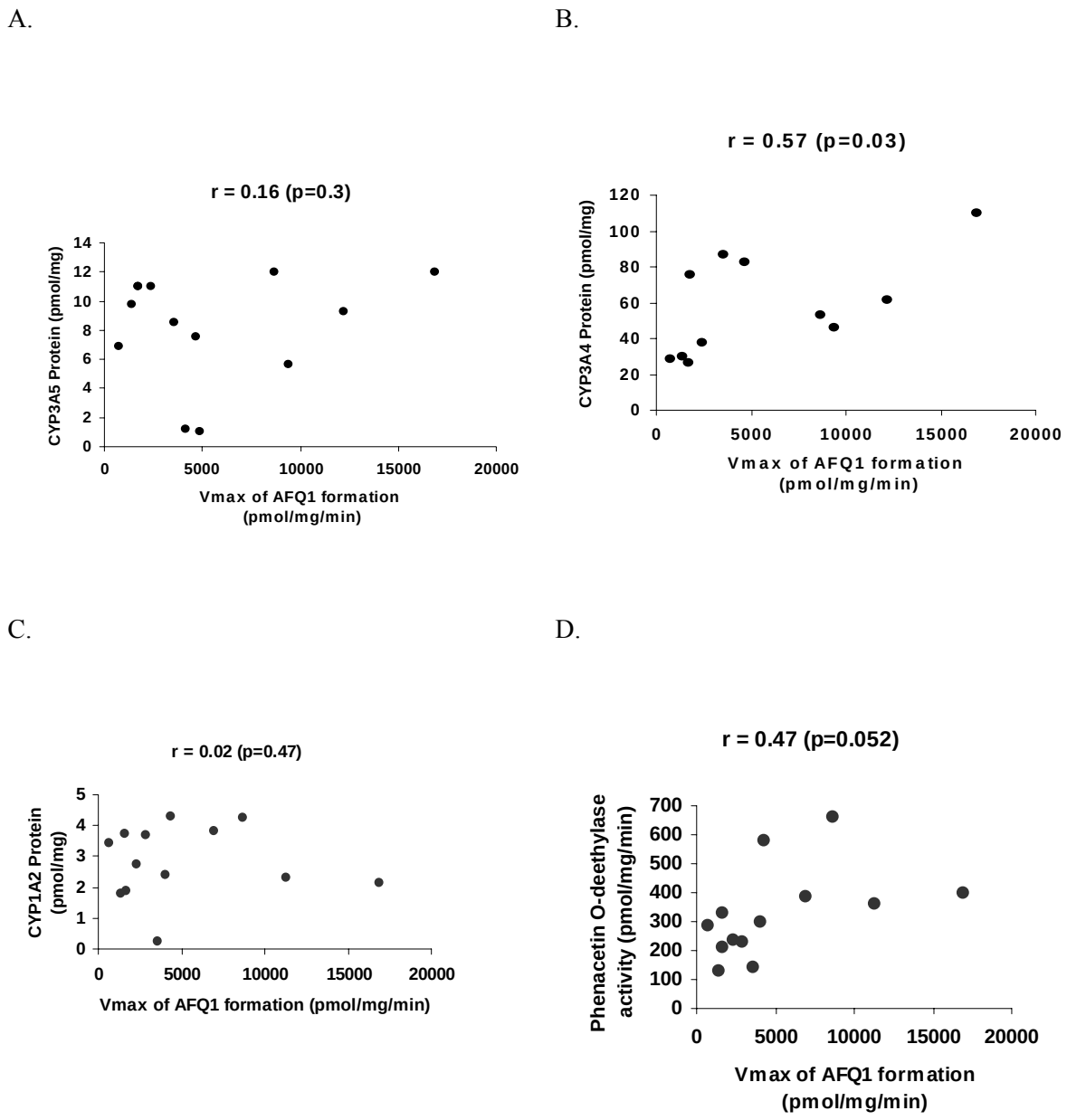
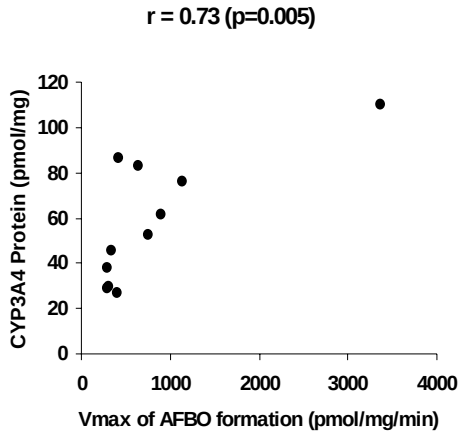
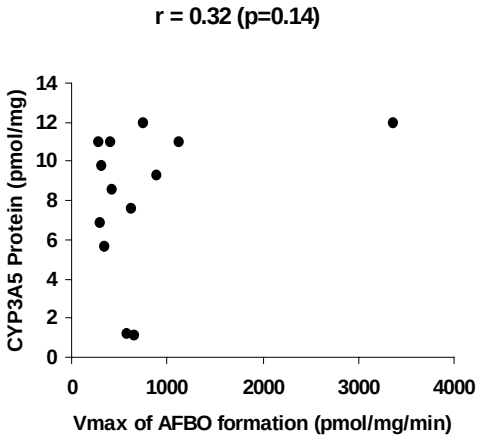


Fig. 23 Correlations between V_{max} of AFQ1 formation and (A) CYP3A5 protein, (B) CYP3A4 protein, (C) CYP1A2 protein, and (D) phenacetin-O-deethylase activity in a panel of human liver microsomes.

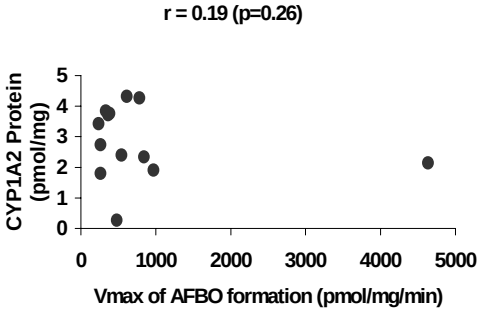
A.



B.



C.



D.

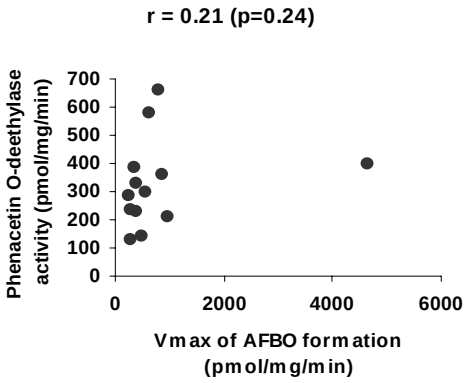


Fig. 24 Correlations between V_{max} of AFBO formation and (A) CYP3A4 protein, (B) CYP3A5 protein, (C) CYP1A2 protein, and (D) phenacetin-O-deethylase activity in a panel of human liver microsomes.

The absence of correlations between the hepatic CYP3A5 and CYP1A2 expression level and AFBO production was in apparent disagreement with the considerable clearance of AFB1 to AFBO by the recombinant CYP3A5 and CYP1A2 (Table 12). To resolve this discrepancy, the contributions of CYP3A5 and of CYP1A2 to AFBO formation were calculated at an epidemiologically relevant AFBO concentration of 0.1 µM using a hepatic abundance model. The model takes into account both the kinetic parameters (Table 12) and the hepatic expression levels of CYP3A5 and CYP1A2 (Table 13). Under these conditions, the contribution of CYP3A5 showed an inverse, statistically significant correlation with the amount of CYP3A4 protein (Fig. 25; $r = 0.82$; $p<0.001$). The highest relative contribution of CYP3A5 to AFBO formation (15.3%) was observed in the liver HH108, which is a CYP3A5 high expresser with the lowest CYP3A4 expression of all livers investigated.

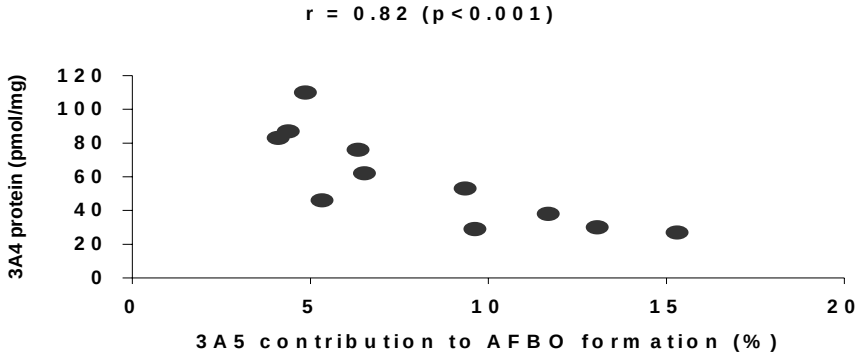


Fig. 25 Correlation between the relative contribution of CYP3A5 to AFBO formation and the expression of the CYP3A4 protein in a panel of human liver microsomes from CYP3A5 high expressers. Data calculated using a hepatic abundance model for the *in vivo* relevant AFB1 concentration of 0.1 µM using kinetics and protein expression data from Tables 12 and 13.

Using the same model, the relative contributions of CYP3A4, CYP3A5, CYP3A7, and CYP1A2 to the primary conversion of AFB1 (0.1 µM) were calculated. On average, 89% of AFB1 are converted to AFQ1 and 11% to AFBO (Fig. 26), but, depending on the liver, the ratio of the two metabolites may vary between 19:1 (95% AFQ1, 5% AFBO) and 1.7:1 (63% AFQ1 and 37% AFBO). CYP3A4 is the exclusive source of AFQ1, except for individuals with increased CYP3A7 expression. In the latter group, CYP3A7 contributes up to 22% of AFQ1. This value results from the specific clearance of AFB1 to AFQ1 by CYP3A7 (Table 12) and the hepatic CYP3A7 expression levels of 24-90 pmol/mg protein (Sim et al., 2005). AFBO is catalyzed by CYP3A4, CYP3A5, CYP1A2, and CYP3A7. A

majority of AFBO is contributed by CYP3A4, but its share varies, depending on the concomitant expression of CYP3A5 and CYP3A7. CYP3A5 may contribute between 4% and 15% of AFBO in CYP3A5 high expressers. The respective value for CYP3A7 high expressers is 5-7%, whereas CYP1A2 contributes less than 5% of AFBO.

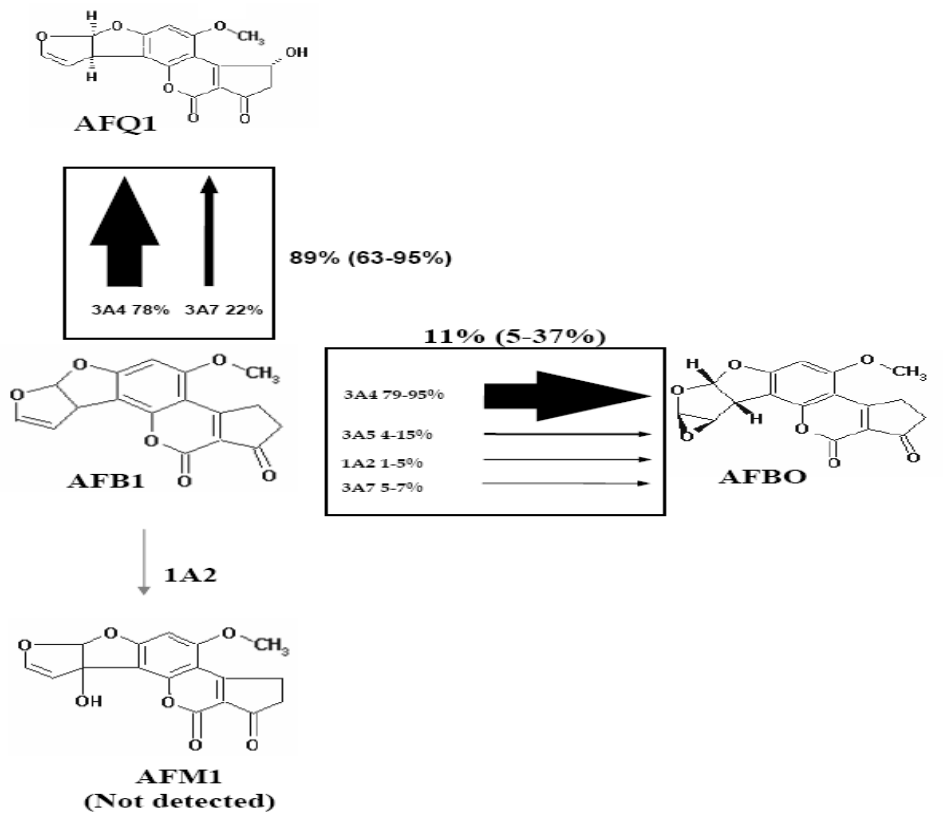


Fig. 26 Hepatic metabolism of AFB1 in CYP3A5 or CYP3A7 high expressers at a concentration of 0.1 µM. Data calculated using a hepatic abundance model for the *in vivo* relevant AFB1 concentration of 0.1 µM using kinetics and protein expression data from Tables 11 and 12. Both the detoxification to AFQ1 and the oxidation to the genotoxic AFBO are catalyzed predominantly by CYP3A4. CYP3A5 contributes between 4 and 15% of AFBO formation, depending on the concomitant expression of CYP3A4. The contribution of CYP3A7 to AFQ1 and AFBO formation in CYP3A7 high expressors (in italics) was calculated using hepatic CYP3A7 expression levels reported by Sim et al. (Sim et al., 2005).

5 DISCUSSION

5.1 Determinants of CYP3A5 activity *in vitro*

It is still disputed whether CYP3A5 activity is similar to or lower than that of CYP3A4 under *in vitro* and *in vivo* conditions, although the substrate specificity and product regioselectivity of CYP3A4 can differ from that of CYP3A5. The activities of CYP3A isozymes had previously been shown to be affected by several chemicals and proteins, but the optimal conditions and the existence of differences between CYP3A4 and CYP3A5 isoforms in substrate or reaction specificity are still a matter of debate. Previous studies compared the metabolic capabilities of CYP3A isoforms expressed under different conditions either in the presence or absence of b5 (Gillam et al., 1995; Williams et al., 2002; Yamazaki et al., 2002; Patki et al., 2003) which hampered the interpretation of the results.

In our hands, using baculovirus-expressed (BE) CYP3A4 and CYP3A5 enzymes, we detected the inhibition of the CYP3A activity by additional exogenous oxidoreductase (OR). This finding suggests that the BE preparations used contained an optimal, but possibly an excess of OR. In the latter case, the true activity of the P450 would be underestimated. Thus it is conceivable that some P450 preparations may contain saturated amounts of OR, at least in some lots. While optimal ratio of P450 to OR can be determined for individually expressed proteins, caution is advised for co-expressed proteins. The same is true for HLM, where testosterone hydroxylation activity was inhibited by either OR or b5. Previously, Yamazaki and colleagues demonstrated an increase of testosterone hydroxylation in HLM supplemented with OR (co-expressed with CYP1A2, which does not metabolize testosterone) (Yamazaki et al., 2002). Together, these observations suggest that the OR and b5 content may vary between different HLM preparations and therefore may falsify the activity levels.

Another important parameter is the expression system used for CYP3A activity assessment. The isozyme-specific activity of CYP3A4 is usually considered several-fold higher than that of CYP3A5, although there are some exceptions to this opinion (Gillam et al., 1995). In our experiments , under similar expression and incubation conditions, CYP3A4 and CYP3A5 appear to exhibit similar specific activities. Furthermore, a co-expression of b5 has a much stronger effect on the CYP3A4 activity than

its addition to the incubation mixture (results, section 4.1 fig. 12). Indeed, upon addition of b5 to CYP3A4 or CYP3A5 co-expressed (BE) with OR, or in the absence of b5, these two P450 exhibit similar activities. The much higher activity of CYP3A4 is observed only in a preparation where all three enzymes are co-expressed (CYP3A4/OR/b5), as previously reported by Williams and colleagues (Williams et al., 2002). This suggests that the method of co-expression rather than the isozyme-specific activity of CYP3A4 are responsible for this high activity. Consequently, we propose that the specific activities of CYP3A4 and CYP3A5 towards testosterone are similar. This may not apply to other substrates, in agreement with CYP3A4 and CYP3A5, K_m and V_{max} values strongly varying with substrate used (table 14, (Williams et al., 2002)). Altogether, these data indicate that comparisons of activities of these isozymes towards other substrates should be performed in strictly comparable expression systems.

Table 14 Enzyme kinetic parameters for testosterone 6ß-hydroxylation, 13-O-demethylation of Tacrolimus and AFB1-8,9-epoxidation catalyzed by BE CYP3A4 and CYP3A5. The testosterone concentration range varied from 50 to 400 µM. The tacrolimus concentration range varied from 0 to 12.5 µM. The AFB1 concentration range varied from 25 to 500 µM. The baculovirus-expressed enzymes were supplemented with cytochrome b5. The ratio of BE CYP3A/OR to b5 was 1:1.

Baculovirus-Expressed Enzyme Systems	Enzyme kinetic parameters		
	K_m (µM)	V_{max} (pmol/pmol CYP/min)	V_{max}/K_m (µl/pmol CYP/min)
Testosterone 6ß-hydroxylation			
CYP3A4/OR + b5	128.8	14.5	0.11
CYP3A5/OR + b5	113.3	13.1	0.11
Tacrolimus 13-O-demethylation			
CYP3A4/OR + b5	1.5	0.72	0.48
CYP3A5/OR + b5	1.4	1.1	0.78
AFB1 8,9-epoxidation			
CYP3A4/OR + b5	130	1.64	0.012
CYP3A5/OR + b5	302	1.2	0.0039

5.2 Contribution of CYP3A5 to the hepatic 6ß-hydroxylation of testosterone

The 6ß hydroxylation of testosterone is a widely used probe of CYP3A activity (Yuan et al., 2002). In our experiments , a majority of the activity (85%) could be inhibited by a monoclonal antibody against CYP3A4 and CYP3A5. Furthermore, the only enzyme capable of testosterone metabolizing with appreciable activity besides CYP3A4, CYP3A5 and CYP3A7 was CYP1A1. This latter enzyme is

usually referred to as extrahepatic. In agreement with numerous recent reports (Nishimura et al., 2002; Chang et al., 2003), we detect CYP1A1 in the liver, although the low transcript numbers suggest low levels of CYP1A1 protein. Altogether, it appears that the 6ß hydroxylation of testosterone is a fairly specific phenotyping probe for CYP3A. However, despite similar specific activities, the contribution of CYP3A4 to the hepatic clearance of testosterone is much more significant than that of CYP3A5. This is evidenced by the poor correlation of V_{max} of testosterone 6ß hydroxylation with CYP3A5 mRNA expression on one side, and a good correlation with CYP3A4 mRNA and protein on the other side. It is obvious that the higher the correlation between an enzyme's expression and a marker enzymatic activity, the higher the contribution of this enzyme to this activity. Of all CYP3A genes, CYP3A4 is the only one that shows an appreciable and statistically significant correlation with the Vmax values of 6ß hydroxylation of testosterone. The correlation with CYP3A5 was poor and remained insignificant despite the consideration of the CYP3A5 polymorphism status. Furthermore, the predicted pharmacokinetic clearances of testosterone did not differ between CYP3A5*3/*3 and CYP3A5*1/*3 livers. The limited contribution of CYP3A5 to the hepatic testosterone 6ß hydroxylation activity in Caucasian liver samples is in agreement with the low share of CYP3A5 transcripts in the CYP3A transcript pool, which is on average 2.5%-3.9% in livers irrespective of the CYP3A5 polymorphism status and does not exceed 10%-20% in livers carrying CYP3A5*1 alleles (Koch et al., 2002; Westlind-Johnsson et al., 2003).

5.3 Contribution of CYP3A5 to the hepatic metabolism of tacrolimus

Previous investigations using human liver microsomes have indicated that CYP3A4 plays an important role in the metabolism of tacrolimus, the major primary metabolite being 13-O-demethyltacrolimus (Sattler et al., 1992; Karanam et al., 1994; Shiraga et al., 1994). It was, however, suggested that members of the CYP3A protein family other than CYP3A4 might be involved in the 13-O demethylation of tacrolimus (Sattler et al., 1992). More recently, Bader et al. (Bader et al., 2000) established that tacrolimus is indeed metabolized by CYP3A5, but no detailed kinetic data were presented.

Using cDNA expressed P450 enzymes we could show that tacrolimus is indeed predominantly metabolized by members of the CYP3A subfamily to 13-O-demethyltacrolimus. The metabolites 13,15-O-demethyltacrolimus and 13,31-O-demethyltacrolimus were also detected, but their amounts were 10-20 times lower than the corresponding levels of 13-0-demethyltacrolimus. Recombinant CYP3A5 metabolized tacrolimus with an affinity comparable to that of CYP3A4, but with a catalytic efficiency (V_{max}/K_m) that was 64% higher than that of CYP3A4, which makes tacrolimus a more specific substrate for CYP3A5 than CYP3A4. This further supports the growing recognition that the specific activities of CYP3A4 and CYP3A5 are comparable for certain substrates including ethylmorphine, lidocaine, testosterone and alfentanil (Gillam et al., 1995; Huang et al., 2004; Kamdem et al., 2004; Klees et al., 2005).

The K_m values observed for recombinant CYP3A4 (1.5 µmol/L) and CYP3A5 (1.4 µmol/L) were similar to the K_m values obtained for the transformation of tacrolimus to 13-0-demethyltacrolimus in a bank of 15 human liver microsomes (median: 0.74 µmol/L; range: 0.38 to 1.20 µmol/L). Using the kinetic constants derived from the recombinant enzyme experiments and the concentrations of CYP3A4 and CYP3A5 protein in the respective microsomes, we were able to estimate the contribution of CYP3A5 to the transformation of tacrolimus in each microsomal preparation. The contribution of CYP3A5 to the 13-O-demethylation of tacrolimus in human liver microsomes varied from 1.5% to 40% and it was especially strong in livers with low CYP3A4, whereas it was lower in those with high CYP3A4 and in the three CYP3A5 “low expressers”. These findings support the observations that CYP3A5 is an important source of CYP3A interindividual variability, when CYP3A5 content represents a significant fraction of the total hepatic CYP3A pool (Huang et al., 2004; Kamdem et al., 2004; Yamaori et al., 2004). Conversely, the share of CYP3A5 in tacrolimus metabolism undergoes “dilution” in individuals with high CYP3A4 expression. In other words, the relative contribution of CYP3A5 strongly depends on the concomitant expression level of CYP3A4. The predominant role of CYP3A isoforms in the 13-O-demethylation of tacrolimus is in agreement with the good correlation observed between tacrolimus 13-O-demethylation activity and 6ß-hydroxylation of testosterone in the liver samples investigated. The latter parameter is widely used as a marker of CYP3A activity (Yuan et al., 2002; Kamdem et al., 2004).

In our experiments , the average predicted tacrolimus pharmacokinetic clearances in CYP3A5 low expressers (3.57 ml/min/kg) and CYP3A5 high expressers (8.60 ml/min/kg) were in general in the same range as *in vivo* (0.68 – 6 ml/min/kg) (Christians et al., 2002). Clearance values >6 ml/min/kg observed in some liver samples may have been caused by the high CYP3A expression.

Taken together, our results explain, at least in part, the recently reported positive association between CYP3A5 genotype and tacrolimus dosage (Macphee et al., 2002; Hesselink et al., 2003; Thervet et al., 2003; Zheng et al., 2003; Haufroid et al., 2004; Zheng et al., 2004; Macphee et al., 2005; Thervet et al., 2005). Furthermore, our data offer an explanation for the substantial variability in tacrolimus dosage independent of CYP3A5 genotype, i.e. that observed within the groups of „high“ and „low“ CYP3A5 expressers (Haufroid et al., 2004). It is likely that a part of this variability is brought about by genotype-independent differences in CYP3A5 expression. Indeed, CYP3A5 expression varied two-fold (range 6.9 to 14 pmol/mg) among the 12 “high expresser” livers. We speculate that an even bigger variability in tacrolimus maintenance dose is caused by differences in CYP3A4 expression. For comparison, the expression of this CYP3A isozyme varied 11-fold (27-300 pmol/mg) within the same set of 12 samples. Further variability may be due to differential inhibition or induction by exogenous substances (Gibbs et al., 1999; Khan et al., 2002; Burk et al., 2004) and due to the expression of CYP3A5 and CYP3A4 in the small intestine (Tsuchiya et al., 2004). This is particularly important for corticosteroids, since these compounds are often used concomitantly with tacrolimus after organ transplantation, and they are substrates, inducers, and inhibitors of CYP3A (Christians et al., 2002). Besides CYP3A, tacrolimus is a substrate for the P-glycoprotein encoded by the MDR1 gene and the trough concentration of tacrolimus is indeed affected by the intestinal expression level of MDR-1 during the first week after transplantation. Subsequently, it is mostly hepatic metabolism that contributes to the clearance of tacrolimus (Goto et al., 2004). A polymorphism in the MDR1 has been associated with tacrolimus clearance (Macphee et al., 2002; Zheng et al., 2004) although these findings have not been confirmed by other investigators (Hesselink et al., 2003; Haufroid et al., 2004; Tsuchiya et al., 2004). Interestingly, MDR1 and CYP3A5 (as well as CYP3A4) share transcriptional activators such as pregnane X receptor (PXR) (Geick et al., 2001; Burk et al., 2004). This latter

mechanism may explain the intriguing findings by Goto et al., who described an association between a MDR1 polymorphism and CYP3A4 expression (Goto et al., 2004).

Tacrolimus emerges as another clinically-relevant substrate of CYP3A5. Previously, Katz et al. (Katz et al., 2004) found that *in vivo* drug disposition of ABT-773, a ketolide antibiotic that is a substrate for both CYP3A and P-glycoprotein, depends on the CYP3A5, but not on the MDR-1 genotype. CYP3A5 expressers showed a higher metabolism of lovastatin, simvastatin and atorvastatin (Kivisto et al., 2001), whereas, no association was found between the polymorphism and efficacy of statins which are not metabolized by CYP3A5 (fluvastatin, pravastatin).

What are the implications of our findings for the currently discussed (Park et al., 2003), prospect of CYP3A5 genotyping in patients treated with tacrolimus? Certainly, CYP3A5 genotyping will not eliminate the need to monitor tacrolimus blood concentrations, since CYP3A4 also contributes to its metabolism and the expression levels of both CYP3A4 and CYP3A5 (Burk et al., 2004) undergo strong modulation by non-genetic (environmental) factors. However, a CYP3A5 genotype-based adjusted initial dosing regimen for tacrolimus, may allow a more rapid and efficient attainment of therapeutic blood levels in the early postoperative period. Indeed, CYP3A5 high expressers had lower mean tacrolimus concentrations during the first week after transplantation and rejection occurred earlier in these patients (MacPhee et al., 2004). Prospective studies are now needed to validate this hypothesis.

5.4 Contribution of CYP3A4, CYP3A5, CYP3A7 and CYP1A2 to the hepatic production of aflatoxin B1-8,9-epoxide

The primary steps of detoxification and carcinogenic activation of AFB1 have been reported to be catalyzed by CYP3A4, CYP3A5, CYP3A7, and CYP1A2. All these enzymes exhibit substantial inter-individual expression variability, which could affect the ratio between the AFB1 detoxification and activation. This ratio is likely an important determinant of the individual risk to AFB1-induced HCC. Our study constitutes a first investigation of the metabolism of AFB1 by these P450s at concentrations encountered in the liver tissue of exposed individuals, i.e. at around 0.1 µM (Wild et al., 1992; Gallagher et al., 1996; Doi et al., 2002).For this purpose, the metabolism of AFB1 was assessed at

concentrations easily measurable with HPLC and adjusted using a hepatic abundance model. This model takes into consideration the specific activities of the individual P450s and their hepatic expression levels, which had to be determined to start with. The AFB1 metabolite spectra and, where available, the kinetic parameters were in agreement with previous reports (Gillam et al., 1995; Gallagher et al., 1996; Ueng et al., 1997; Wang et al., 1998). AFBO was the exclusive AFB1 product catalyzed by the recombinant CYP3A5, but it was also catalyzed by CYP3A4, CYP3A7, and CYP1A2. AFBO is a mix of the genotoxic AFB1-8,9-exo-epoxide and the less toxic stereoisomer AFB1-endo-8,9-epoxide, but it is a fair measure of the AFB1 carcinogenic activation (Ueng et al., 1995). AFQ1, the major detoxification product, was catalyzed by CYP3A4 and CYP3A7, whereas AFM1 was specific for CYP1A2. The extent of CYP3A4 variability in HLMs was smaller than in some previous studies, but it was in the same range as that recently reported by Sim et al. (Sim et al., 2005). It has been suggested that the variability in the expression of CYP3A4 may have been overestimated in earlier studies, due to tissue collection artefacts (Floyd et al., 2003). The expression of CYP3A5 was bimodal, as reported previously (Hustert et al., 2001; Kuehl et al., 2001). To allow for a first estimate of the contribution of CYP3A7 to AFB1 metabolism, we used CYP3A7 expression levels measured in human livers with a recently developed, specific CYP3A7 antibody (Sim et al., 2005).

For the epidemiologically-relevant AFB1 concentration of 0.1 μM, our data predict a substantial inter-individual variability of the toxin's disposition. The share of AFB1 converted to the carcinogenic AFBO varies between 5% and 37% even in this relatively small set of HLMs. Correspondingly the rate widely (from 19:1 to 1.7:1) varies between AFBO and AFQ1, the primary detoxification product of AFB1. The hepatic abundance model predicts that a majority of both AFBO and AFQ1 is catalyzed by CYP3A4. This is supported by the high correlation coefficients between the HLM expression levels of CYP3A4 and V_{max} values of, respectively, AFBO and AFQ1 production. It is also in agreement with the high correlation between V_{max} values of AFBO and AFQ1 formations measured in HLMs, when taking into account the similarity of AFBO and AFQ1 kinetics in recombinant CYP3A4 and HLMs.

Although recombinant CYP3A7 also generates AFQ1, its extent is negligible in most individuals in comparison to CYP3A4, since they express only traces of CYP3A7 (Sim et al., 2005). In CYP3A7 high expressers, which account for approximately 15% of Caucasians (Sim et al., 2005), this P450 may contribute on average almost a quarter of AFQ1 production. This estimate is based on CYP3A7 accounting for almost a quarter of the total CYP3A in CYP3A7 high expressers (Sim et al., 2005), combined with similar specific AFB1 clearances of CYP3A4 and CYP3A7 towards AFQ1. The frequencies of the CYP3A7 polymorphism in populations exposed to high AFB1 concentrations such as Asians and Africans are currently unknown.

A dominant role of CYP3A4 in AFBO production is in agreement with the findings of Doi and colleagues, who detected a correlation approaching statistical significance between CYP3A4 expression and AFB1-DNA, adduct levels in a panel of adult human livers (Doi et al., 2002). In addition to CYP3A4, AFBO production is contributed to by CYP3A5, CYP3A7, and CYP1A2, which may affect the share of AFB1 converted to AFBO. The contribution of CYP3A7 in CYP3A7 high expressers (Sim et al., 2005) is estimated to be between 5% and 7%, whereas that of CYP1A2 is between 1% and 5%. CYP3A5 may contribute up to 15.3% of AFBO in CYP3A5 high expressers. This value is still relatively modest in comparison with CYP3A4 (79-95%) and it is consistent with the low value of the correlation coefficient between the CYP3A5 protein expression level and V_{max} of AFBO production in the HLMs investigated. On the other side, we found a significant, inverse correlation between the contribution of CYP3A5 to the hepatic AFBO production, and the expression of the CYP3A4 protein. This means that the relative contribution of CYP3A5 to AFBO production is highly dependent on the concomitant expression of CYP3A4. This is in agreement with the recent observation of AFB1-albumin adducts blood plasma levels increased especially strongly in AFB1-exposed CYP3A5 high expressers with low concomitant CYP3A4 expression (Wojnowski et al., 2004). Similar relationships have been reported for several other substrates common to CYP3A4 and CYP3A5, with the effect of the CYP3A5 polymorphism especially pronounced against a background of low CYP3A4 expression (Huang et al., 2004; Kamdem et al., 2004; Kamdem et al., 2005).In summary, these findings indicate that, following CYP3A4, CYP3A5 expression status is the second-most important determinant of the carcinogenic AFB1 activation to AFBO. The clinical importance of

the CYP3A5 effect on AFB1 metabolism will be ultimately determined by genotyping HCC patients from areas of high AFB1 exposure.

We detected in our microsomal incubations no AFM1, which is an AFB1 metabolite specific for CYP1A2. Usually considered a detoxification product, AFM1 can be converted, e.g. by cultured liver cells, to the genotoxic AFM-8,9-epoxide (Loveland et al., 1988; Bujons et al., 1995; Neal et al., 1998). Such a conversion could be responsible for the absence of AFM1 from our incubations of HLMs with AFB1. Alternatively, the production of AFM1 may have been simply too low to be detected. In agreements with this latter interpretation, the production of AFM1 by HLMs was a magnitude smaller that that of AFQ1 in the available reports on hepatic AFM1 production (Ramsdell and Eaton, 1990; Ramsdell et al., 1991). Likewise, median AFM1 concentrations in urine and feces of AFB1-exposed Chinese individuals accounted, respectively, for $1/60^{th}$ and $1/260^{th}$ of the corresponding AFQ1 values (Mykkanen et al., 2005). In the same study, urinary excretion of AFQ1 (but not that of AFM1) correlated significantly with the excretion of the AFB1-N^7-guanine adduct, which is considered to be the best predictor of hepatocellular carcinoma risk (Groopman et al., 1992; Qian et al., 1994; Groopman et al., 1996). Furthermore, Doi and colleagues (Doi et al., 2002) detected no correlation between CYP1A2 expression level and AFB1-DNA adducts in the same panel of microsomes from 10 human livers in which they reported a correlation approaching statistical significance between these adducts and CYP3A4 expression. In conclusion, all these data suggest that the disposition of AFB1 to AFM1 in HLMs may be negligible.

Similarly negligible may be the contribution of CYP1A2 to the formation of AFBO. Our conclusion that CYP3A4 is responsible for the majority of AFBO formation in human livers is in agreement with expectations from several studies with purified or recombinant P450 (Shimada and Guengerich, 1989; Forrester et al., 1990; Guengerich and Kim, 1990; Raney et al., 1992a; Raney et al., 1992b; Raney et al., 1992c; Gillam et al., 1995). It is in opposition to the only work which assessed AFB1 metabolism in microsomes from human livers (Gallagher et al., 1996). These authors predicted that, at AFB1 concentrations encountered *in vivo*, over 99% of the hepatic AFBO production would be contributed by CYP1A2. However, the critically important hepatic expression levels of CYP1A2 and CYP3A4 were not considered in the calculation. The postulated dominant role of CYP1A2 in AFBO production

was also not supported by: 1) V_{max} of AFBO production, which was highest in the liver with the highest CYP3A4 expression and not in the liver with the highest CYP1A2 expression, as would be expected (Table2 in (Gallagher et al., 1996) and 2) the finding that only 20-46% of AFBO production was inhibited by the specific CYP1A2 inhibitor furafylline (compare Tables 1 and 2 in (Gallagher et al., 1996)).

Could our findings have any immediate implications for public health decisions in areas with high prevalence of AFB1-associated HCC? Preventive measures such as vaccination or chemoprotection are expected to reduce the burden of HCC especially effectively if they are applied to individuals at particularly high risk. Since all AFB1 metabolizing enzymes are expressed in the liver at individually variable levels, targeted chemoprotection by P450 inhibition represents a valid hypothesis (Kensler et al., 2003). Our results argue against targeting any preventive measures specifically to CYP3A5, CYP3A7, or CYP1A2 high expressers. Considering the prominent contribution of CYP3A4 to AFBO formation, much more effective should be the inhibition of CYP3A4 in individuals with particularly high hepatic levels of this P450. However, this would require genetic or phenotypic CYP3A4 activity markers, the development of which has proven difficult. More practical would be the reduction of CYP3A4 expression by avoiding pharmacological and dietary CYP3A4 inducers. These substances can sometimes enhance the turnover of CYP3A4 drug substrates to an extent where their effectiveness is abolished (Lucey et al., 1990) and they likely increase AFBO levels in individuals exposed to AFB1. Preventing CYP3A4 induction would have the added benefit of a similar effect on CYP3A5 and CYP3A7 in the respective high expressors of these P450. Indeed, all three CYP3A genes exhibit overlaps in the spectra of inducers, due to similarities in their transcription regulation (Burk et al., 2002; Burk et al., 2004).

6 CONCLUDING REMARKS

Three substrates (Testosterone, Aflatoxin B1 and Tacrolimus) have been used to investigate the impact of CYP3A5 genetic polymorphism on biotransformation. The main conclusions from this investigation are:

1. Under similar expression and incubation conditions, CYP3A4 and CYP3A5 exhibit similar specific activities and are similarly dependent on cytochrome b5.
2. On average, the contribution of CYP3A5 to 6ß-hydroxylation of testosterone in Caucasian livers is limited (3%), due to the much lower expression levels of CYP3A5 in comparison to CYP3A4. However, it may approach 5-54% in CYP3A5 high expressers with concomitant low expression of CYP3A4.
3. CYP3A5 is an important source of variability in the hepatic clearance of tacrolimus, thus explaining the positive association observed between CYP3A5 genotype and tacrolimus dosage. The importance of CYP3A5 for tacrolimus clearance is also dependent on the concomitant CYP3A4 activity.
4. CYP3A4 contributed a majority of AFBO and AFQ1 and its expression level was the most important determinant of the AFB1 disposition towards these primary metabolites. CYP3A5, which exclusively produced AFBO, was the second-most important enzyme activating AFB1 to AFBO, followed by CYP3A7 and CYP1A2.

Altogether, these findings suggest that CYP3A5 is an important source of CYP3A interindividual variability, when CYP3A5 content represents a significant fraction of the total hepatic CYP3A pool.

7 FUTURE PERSPECTIVES

Among patients differences in drug responses are common and often lead to challenges in optimizing a dosage regimen for an individual patient. Such variability in drug response is multifactorial, including environmental, disease state and genetic factors that affect drug disposition. Pharmacogenetics is one of the fields of clinical pharmacology, which studies how genetic factors influence drug response. Today, the relationship between dosage requirements and pharmacogenetic polymorphisms in drug metabolizing enzymes are best substantiated for cytochromes such as CYP2D6 (antidepressants, antipsychotics, analgesics) (Kirchheiner et al., 2004; Lotsch et al., 2004; Ingelman-Sundberg, 2005), CYP2C9 (warfarine, antidiabetics) (Kirchheiner and Brockmoller, 2005)), CYP2C19 (diazepam, proton pump inhibitors) (Goldstein, 2001; Desta et al., 2002; Wilkinson, 2005) and CYP2B6 (efavirenz) (Haas et al., 2004; Rotger et al., 2005).

CYP3A plays a dominant role in the metabolic elimination of more xeno- and endobiotics than any other biotransformation enzyme and the pharmacogenetic determinants of CYP3A interindividual differences have been identified. The most frequent ones are found in CYP3A5 and CYP3A7, which are represented by CYP3A5*1 and the newly identified CYP3A7*2 genetic variants, respectively. CYP3A4*1B, the only common CYP3A4 variant does not appear to substantially affect the CYP3A4 function *in vitro* and *in vivo*; and is in linkage disequilibrium with CYP3A5*1 allele. Whereas the clinical relevance of CYP3A7 and CYP3A43 polymorphisms remains to be assessed, the polymorphic expression of CYP3A5 provides a possible explanation for the lower CYP3A-mediated metabolism reported in some individuals when compared to others. However, the penetrance of the CYP3A5 polymorphism is likely to be compromised by the concomitant expression of CYP3A4, although its relevance may be higher in organs devoid of CYP3A4 expression, such as kidney. Therefore, these markers will unlikely lead to any clinical application as long as there are no markers predictive of CYP3A4 expression. As mentioned above, the expressivity of the CYP3A5 polymorphism in relation to drug metabolism strongly depends on the concomitant expression of CYP3A4, and also this will likely be the case for the CYP3A7 polymorphism and CYP3A4. Possible exceptions to the rule could be drugs predominantly metabolized by either CYP3A7 or CYP3A5, but not by CYP3A4. At present,

the most promising candidate for such a drug is tacrolimus, where almost half of the dose variability can be explained via the CYP3A5 polymorphism status. The expressivity of the CYP3A5 polymorphism could also be sufficient in the absence of substrate specificity, e.g. in the kidney, which expresses only very low levels of CYP3A4 and CYP3A7. Nevertheless, the emerging associations with *CYP3A5*1* alleles are unlikely to lead to routine clinical genotyping of this variant in the near future. This assessment is first of all based on the clinical application of some other polymorphisms in drug metabolizing enzymes, which have been known for a long time while this knowledge has not been translated into clinical applications. This has been mostly due to the lack of clinical studies demonstrating the clinical value of genotyping using the criteria of evidence-based medicine. This situation may change dramatically, depending on the results of several ongoing large prospective studies, e.g. on the anticoagulant warfarine and the CYP2C9 polymorphism, but this will take several years. The development and validation of markers predictive of CYP3A expression is likely to take even longer, especially if we ultimately fail to develop markers predictive for CYP3A4. In this case, it may still be possible to reduce drug interactions involving CYP3A by pharmacological means. Cyclosporine has been administered in some countries together with CYP3A inhibitors, such as ketoconazole or diltiazem. This procedure has allowed for a reduction of this drug's dose by up to 85% without any effects on efficacy or toxicity (Martin et al., 1999). Similar strategies could be applied to other drugs that are metabolized by CYP3A and have a narrow therapeutic index.

8 SUMMARY

Enzymes of the cytochrome P450 (CYP) subfamily 3A comprise the largest portion of the liver and small intestinal CYP protein and they are involved in the metabolism of 45-60% of all currently used drugs. In addition to drugs, CYP3A isozymes metabolize a variety of other compounds including steroid hormones, toxins and carcinogens. It is also well known that the hepatic expression and activity of CYP3A isozymes varies from individual to individual. Unlike CYP3A4, the homologous enzyme CYP3A5 is polymorphically expressed. Several genetic variants, of which CYP3A5*3 is the only one found in all ethnic groups, can reduce the expression of CYP3A5 to less than 1/1000 of that found in carriers of the wild type allele (CYP3A5*1).

To investigate the significance of CYP3A5 genetic polymorphism in the metabolism of drugs and carcinogens, we assessed the relative contribution of CYP3A5 to the 6ß-hydroxylation of testosterone (steroid hormone), to the 13-O demethylation of tacrolimus (an immunosuppressive drug), and to the 8,9-epoxidation of AFB1 (carcinogen) using cDNA-expressed enzymes and a bank of human liver microsomes derived from low and high CYP3A5 expressers.

In our experiments, under similar expression and incubation conditions, CYP3A4 and CYP3A5 exhibit similar specific activities toward testosterone. A co-expression of cytochrome b5 (b5) has a much stronger effect on the CYP3A4 activity than its addition to the incubation mixture. For 6ß-hydroxytestoterone formation the calculated K_m and V_{max} values for CYP3A4 and CYP3A5 cDNA-expressed microsomes were 129 µM and 14.5 pmol/pmol CYP/min, 114 µM and 13.1 pmol/pmol CYP/min, respectively. With human liver microsomes (HLM), the CYP3A5 low expressers had a mean testosterone 6ß-hydroxylation V_{max} value of 3798 pmol/mg/min, compared with 2976 pmol/mg/min in CYP3A5 high expressers (statistically not a significant difference). The CYP3A5 contribution to 6ß-hydroxylation of testosterone accounted on average for 30% in CYP3A5 high expresser livers.

Recombinant CYP3A5 metabolized tacrolimus with an affinity comparable to that of CYP3A4, but with an intrinsic clearance (V_{max}/K_m) that was 64% higher than that of CYP3A4. The calculated K_m and V_{max} values (13-O-demethyltacrolimus formation) for CYP3A4 versus CYP3A5 cDNA-expressed microsomes were 1.5 µM and 0.72 pmol/pmol CYP/min, 1.4 µM versus 1.1 pmol/pmol CYP/min,

respectively. With HLM, the CYP3A5 low expressers had a mean tacrolimus 13-O demethylation V_{max} value of 285 pmol/mg/min, compared with 981 pmol/mg/min in CYP3A5 high expressers. The contribution of CYP3A5 to 13-O-demethylation of tacrolimus in human liver microsomes varied from 1.5% to 40% and was particularly strong in livers with low CYP3A4, whereas it was lower in those with high CYP3A4.

The calculated K_m and V_{max} values (AFB1-8,9-epoxide formation) for CYP3A4 versus CYP3A5 cDNA-expressed microsomes were 130 µM and 1.64 pmol/pmol CYP/min, 302 µM versus 1.2 pmol/pmol CYP/min, respectively. With HLM, the CYP3A5 low expressers had a mean AFB1-8,9-epoxidation V_{max} value of 545 pmol/mg/min, compared with 873 pmol/mg/min in CYP3A5 high expressers. The relative contribution of CYP3A5 to AFBO formation strongly depended on the expression of CYP3A4 and it was as high as 15% in a CYP3A5 high expresser with the lowest amount of CYP3A4 protein of all livers.

Altogether, these findings demonstrate that CYP3A5 is an important source of CYP3A interindividual variability, when CYP3A5 represents a significant fraction of the total hepatic CYP3A pool. Among the CYP3A substrates analyzed in the present work, the impact of CYP3A5 was highest for the immunosuppressant tacrolimus.

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10 CURRICULUM VITAE

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